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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Li et al.		
Application No.:	09/817,538	Group:	1635
Filed:	March 26, 2001	Examiner:	K. Lacourciere
Confirmation No.:	6847		
For:	ANTISENSE OLIGONUCLEOTIDE INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS		

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Commission for Patents
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APPEAL BRIEF

Hon. Assistant Commissioner for Patents:

Applicant hereby appeals from the final rejection of Claims 1-3 and 5 of the above identified patent application.

I. Real Party of Interest

The present application is wholly owned by MethylGene, Inc., by assignment by the inventors as recorded at Reel/Frame 012081/0968.

II. Related Appeals and Interferences

There are no current appeals or interferences which would directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. Status of Claims

Claims 1-36 were originally filed in the instant application. Claims 4, 6 and 8-36 were canceled during prosecution without prejudice. Claims 1-3 and 5 are currently pending in this Appeal.

IV. Status of Amendments

Claim 7 was amended subsequent to the Final rejection to correct the recitation of the claim to which Claim 7 depends upon. This amendment was entered by the Examiner in the Advisory Action mailed from the U.S. Patent and Trademark Office (USPTO) on September 25, 2003. Appellants also acknowledge that in the Office Action mailed from the USPTO on May 6, 2003, the Examiner found Claim 7, as amended, to be free of prior art.

V. Summary of Invention

The invention relates to the inhibition of histone deacetylase expression. Specifically, the invention provides oligonucleotides that inhibit one or more histone deacetylase isoforms, but less than all histone deacetylase isoforms, by inhibiting expression at the nucleic acid level (See Detailed Description at page 15, line 14 to page 16, line 4). The claimed oligonucleotide can be a chimeric or hybrid oligonucleotide and have a variety of modifications (See Detailed Description at page 18, line 5 to page 20, line 9).

VI. Issues

The sole issue to be determined in this Appeal is whether Claims 1-3 and 5 are unpatentable over Yoshida et al. in view of the collection of Taylor *et al.*, (DDT vol. 4, No. 12, 12/12/99, pages 562-567), Bennett *et al.*, (Chapter 2, pages 13-46, from Methods in Molecular Medicine: Antisense Therapeutics, 1996), Baracchini *et al.*, (U.S. Patent 5,801,154), Cowsert (U.S. Patent 5,951,455) and the sequence of HDAC-1 (GenBank Accession No. U50079).

VII. Argument

Claims 1-3 and 5 are rejected as being unpatentable over Yoshida *et al.*, (hereinafter "Yoshida"), in view of the collection of Taylor *et al.*, (DDT vol. 4, No. 12, 12/12/99, pages 562-567), Bennett *et al.*, (Chapter 2, pages 13-46, from Methods in Molecular Medicine:

Antisense Therapeutics, 1996), Baracchini *et al.*, (U.S. Patent 5,801,154), Cowsert (U.S. Patent 5,951,455) and the sequence of HDAC-1 (GenBank Accession No. U50079).

As previously argued, the combination of Yoshida and others fails to render the claimed invention obvious because there is no motivation or suggestion, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. According to the Examiner, the motivation to combine Yoshida with the other references is that Yoshida taught the need for "the use of a **more specific and potent inhibitor** of histone deacetylase... to carry out further more refined analysis" (emphasis added). However, the Examiner continues to take this statement out of context.

Yoshida describes that prior to their publication, n-butyrate, the only available small molecule inhibitor of histone deacetylase, produced pleitrophic effects on other enzymes, cytoskeleton, cell membranes, etc. Thus, the technical problem faced by Yoshida was to find a more specific and potent inhibitor of histone deacetylase than n-butyrate. Yoshida solved this problem by using another small molecule inhibitor of histone deacetylase refered to as (R)-Trichostatin A (hereinafter "TSA").

Yoshida describes a technical problem and its solution. Yet the Examiner maintains that Yoshida not only provides the motivation to find further "more specific and potent inhibitors" of histone deacetylase but also provides the motivation to look outside the small molecule inhibitor art to antisense technology even though (a) TSA is the more specific and potent inhibitor Yoshida describes a need for and (b) there is no mention, explicitly or implicitly, within Yoshida that antisense technology could be used to provide specific and potent inhibitors of histone deacetylase. Therefore, Yoshida, at best, provides the motivation to find a better "small molecule" inhibitor of histone deacetylase or to optimize TSA. However, it would even be a stretch to find the motivation to do this, either explicitly or implicitly, within Yoshida.

Further evidence of the Yoshida's lack of motivation to go outside the small molecule art is demonstrated in a subsequent article by Yoshida (IDS Reference A1, IDS filed 10/09/01) published three (3) years after the article cited in the Office Action. In this article Yoshida still teaches the use of TSA as a more specific and potent inhibitor of histone deacetylase with no mention, either explicitly or implicitly, to look outside the small molecule art. Additionally, a review of papers that have cited Yoshida was undertaken and none of them were found to mention or suggest the use of antisense technology to inhibit histone deacetylase. For example, Furumai et al (2001) PNAS 98(1):87-92 (attached hereto as Exhibit 1), only teach small molecule inhibitors and specifically state that CHAP, their small molecule, is a unique lead to develop isoform-specific HDAC inhibitors (end of Abstract). There is no mention or suggestion of antisense technology in the least. Saito et al. (1999) PNAS 96:4592-4597 (attached hereto as Exhibit 2), also only teach small molecule inhibitors, and again, no teaching or suggestion of antisense technology. Marks et al. (2000) Journal of the National Cancer Institute 92(15):1210-1216 (attached hereto as Exhibit 3), is a review of HDAC inhibitors, and as shown in the section entitled "HDAC Inhibitors" (beginning on page 1212) only small molecule inhibitors are taught and there is no mention or suggestion of antisense technology to inhibit HDAC. Marks et al. (2001) Clinical Cancer Research 7:759-760 (attached hereto as Exhibit 4), which is an editorial on HDAC inhibitors, reviews types of HDAC inhibitors and again there is no mention or suggestion of antisense technology. Kwon et al (1998) PNAS 95: 3356-3361 (attached hereto as Exhibit 5), teaches that new methods in small molecule synthesis and screening will provide a useful guide to HDAC research efforts. There is not mention or suggestion of using antisense technology. Moreover, Selker et al (1998) PNAS 95:9430-9435 (attached hereto as Exhibit 6), demonstrates that even in 1998, 8 years after the Yoshida article (ref # 18), TSA is still the drug of choice and therefore it was not obvious to develop antisense inhibitors.

These references show that Yoshida does not lead one of skill in the art to look in the direction of antisense technology to develop an HDAC inhibitor and thus it would not have been obvious to do so having knowledge of the teachings of Yoshida. In fact, the combined teachings of these documents actually teach away from developing antisense inhibitors.

An Examiner is not free to pick and choose references in an attempt to provide the teaching or suggestion of all the claimed limitations of the instant invention. The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination, and the level of skill in the art cannot be relied upon to provide the suggestion to combine references (See Al-Site Corp. v. VSI Int'l Inc., 174 F.3d 1308, (Fed. Cir. 1999)). Yoshida describes the problem of using the small molecule inhibitor n-butyrate and teaches the solution to the problem through the use of another small molecule inhibitor, TSA. Yoshida provides no motivation or suggestion regarding the desirability to look for further inhibitors of histone deacetylase, much less to look for such inhibitors in the alternative art of antisense technology. The need to combine five references to maintain this rejection further emphasizes this point.

Accordingly, Appellants respectfully reiterate that there is no motivation in the prior art to combine the cited references. Thus, Appellants respectfully request that the rejection of Claims 1-3 and 5 for obviousness be withdrawn.

Respectfully submitted,

Dated: November 5 2004

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Appendix

MET-021US2 – Claims on Appeal

Claim Listing:

1. (Previously Presented) An oligonucleotide having a nucleotide sequence of from 15 to about 26 nucleotides and having one or more phosphorothioate internucleoside linkage, that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA that encodes a portion of HDAC-1 (SEQ ID NO: 2).

2. (Original) The oligonucleotide according to claim 1, wherein the oligonucleotide is a chimeric oligonucleotide.

3. (Original) The oligonucleotide according to claim 1, wherein the oligonucleotide is a hybrid oligonucleotide.

4. CANCELLED.

5. (Previously Presented) The oligonucleotide according to claim 1, being 20-26 nucleotides in length, and being modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2'-O-methyl groups attached to their sugar residues.

6. CANCELLED.

7. (Previously Presented) The oligonucleotide according to claim 1 that is SEQ ID NO: 17 or SEQ ID NO: 18.

8-36 CANCELLED.

Exhibit 1

Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin

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Edited by Paul A. Marks, Memorial Sloan-Kettering Cancer Center, New York, NY, and approved November 3, 2000 (received for review August 22, 2000)

Trichostatin A (TSA) and trapoxin (TPX) are potent inhibitors of histone deacetylases (HDACs). TSA is proposed to block the catalytic reaction by chelating a zinc ion in the active-site pocket through its hydroxamic acid group. On the other hand, the epoxyketone is suggested to be the functional group of TPX capable of alkylating the enzyme. We synthesized a novel TPX analogue containing a hydroxamic acid instead of the epoxyketone. The hybrid compound cyclic hydroxamic acid-containing peptide (CHAP) 1 inhibited HDAC1 at low nanomolar concentrations. The HDAC1 inhibition by CHAP1 was reversible as it was by TSA, in contrast to the irreversible inhibition by TPX. CHAP with an aliphatic chain length of five, which corresponded to that of acetylated lysine, was stronger than those with other lengths. These results suggest that TPX is a substrate mimic and that the replacement of the epoxyketone with the hydroxamic acid converted TPX to an inhibitor chelating the zinc like TSA. Interestingly, HDAC6, but not HDAC1 or HDAC4, was resistant to TPX and CHAP1, whereas TSA inhibited these HDACs to a similar extent. HDAC6 inhibition by TPX at a high concentration was reversible, probably because HDAC6 is not alkylated by TPX. We further synthesized the counterparts of all known naturally occurring cyclic tetrapeptides containing the epoxyketone. HDAC1 was highly sensitive to all these CHAPs much more than HDAC6, indicating that the structure of the cyclic tetrapeptide framework affects the target enzyme specificity. These results suggest that CHAP is a unique lead to develop isoform-specific HDAC inhibitors.

Reversible histone acetylation, which occurs at the ε-amino group of conserved lysine residues clustered near the amino terminus of core histones, mediates changes in nucleosome conformation, which is important in the regulation of gene expression (1). The correlation between acetylation and increased transcription has been known for many years. Highly acetylated nucleosomes are associated with transcriptionally active chromatin, whereas hypoacetylated histones are often found in inactive chromatin. Recent discovery of the enzymes controlling histone acetylation and deacetylation showed that acetylation of histones is an important step in transcription (2, 3). Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HATs were identified to be transcriptional coactivators including GCN5 (4), CREB-binding protein (CBP)/p300 (5), and p300/CBP-associated factor (PCAF; ref. 6), as well as the p160 family proteins. On the other hand, HDACs were found as yeast transcriptional regulators related to Rpd3 (7), Hda1 (8), and Sir2 (9). A number of transcriptional repressors and corepressors, such as Sin3, silencing mediator of retinoid acid and thyroid hormone receptor (SMRT), and nuclear receptor corepressor (N-CoR), were shown to recruit the HDAC complex to the promoter regions (10, 11). To date, at least nine different mammalian HDACs were described, which are classified into three classes, i.e., class I (related to Rpd3; refs. 7, 12–14), class II (related to Hda1; refs. 15–18), and the Sir2 family, the activity of which depends on nicotinamide-adenine dinucleotide (NAD; ref. 9). Specific roles of these enzymes and their target genes still remain to be elucidated.

We have identified HDACs as the target of trichostatin A (TSA) and trapoxin (TPX), both of which are microbial metabolites that induce cell differentiation, cell cycle arrest, and reversal of transformed cells morphology (19). Several phytopathogenic and antifungal compounds related to TPX, such as chlamydocin and HC-toxin, have also been shown to inhibit HDAC (20). Because aberrant histone acetylation has been linked to malignant diseases in some cases, HDAC inhibitors bear great potential as new drugs because of their ability to modulate transcription and to induce differentiation and apoptosis (21). In fact, FK228 (22) and MS-275 (23), potent antitumor agents under clinical investigation, were shown to inhibit HDACs. Recent crystallographic studies (24) showed the zinc-dependent acetamide cleavage reaction by a bacterial enzyme related to HDAC [histone deacetylase-like protein (HDLP)]. Cocrystallization of this enzyme with TSA or suberoylanilide hydroxamic acid (25) demonstrated that these inhibitors mimic the substrate and that chelation of the zinc in the catalytic pocket by the hydroxamic acid group is the main mechanism of inhibition (24). Conservation of the amino acid sequences of the loops that form the active-site pocket among HDLP and class I and class II HDACs strongly suggests that HDACs present the same catalytic reaction and TSA inhibition as HDLP. In the case of TPX, we previously showed that the epoxyketone group at the terminus of the side chain of (2S,9S)-2-amino-9,10-epoxy-8-oxodecanoic acid (Aoe) is the enzyme-inhibiting group, causing irreversible inhibition (26). Because TSA can compete with TPX for binding to HDAC1 (7), it seems likely that the aliphatic chain of Aoe also acts as a substrate analogue. If this likelihood is the case, then replacement of the epoxyketone group of TPX

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Aoe, (2S, 9S)-2-amino-9, 10-epoxy-8-oxodecanoic acid; Asu, α-aminosuberic acid; CHAP, cyclic hydroxamic-acid-containing peptide; HDAC, histone deacetylase; HDLP, histone deacetylase-like protein; Pip, pipecolic acid; TPX, trapoxin; TSA, trichostatin A; Tyr(Me), *O*-methyltyrosine; AUT, acid/urea/Triton.

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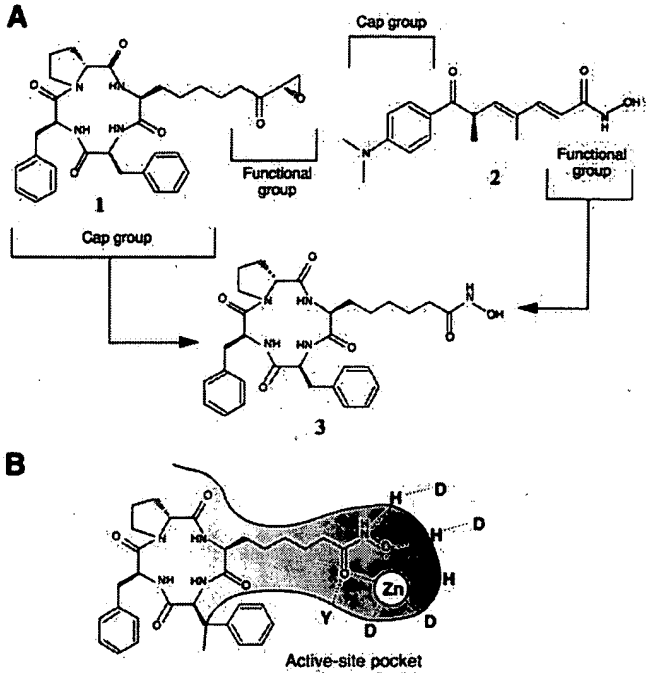

Fig. 1. Molecular design of a TPX/TSA hybrid HDAC inhibitor. (*A*) TPX (**1**) and TSA (**2**) are proposed to consist of cap groups, cyclic tetrapeptide and dimethylamino-phenyl groups, and functional groups for enzyme inhibition, an epoxyketone and a hydroxamic acid, respectively (21, 24). An inhibitor consisting of the cyclic tetrapeptide of TPX B and the hydroxamic acid of TSA (CHAP1) was synthesized (**3**). (*B*) A model for HDAC inhibition by CHAP1. The aliphatic chain with the hydroxamic acid group may be inserted into the tube-like active-site pocket of HDAC, thereby chelating the zinc ion by the hydroxamic acid group at the bottom of the pocket. The cyclic tetrapeptide portion may act as a cap to pack the molecule at the rim of the pocket. The conserved active-site residues revealed by the crystallographic studies (24) are shown as single letters.

(compound **1**) with the hydroxamic acid group of TSA (compound **2**) should convert it to a reversible-type inhibitor that chelates the catalytic center zinc (Fig. 1).

In this paper, we show that a derivative of TPX named cyclic hydroxamic acid-containing peptide (CHAP) 1, in which Aoe in TPX B is replaced by Asu(NHOH) (Fig. 1), strongly and reversibly inhibits HDAC1. Structure-activity relationship study using HDAC1, HDAC4, and HDAC6 showed that the cyclic tetrapeptide portion affects both the enzyme inhibitory potency and specificity. CHAP1 will be a unique lead for development of specific HDAC inhibitors.

Materials and Methods

Synthesis of CHAPs. The cyclic tetrapeptides for CHAPs were prepared according to one of the following three strategies: liquid phase synthesis of linear peptides followed by liquid phase cyclization (strategy 1); solid phase synthesis of linear peptides followed by cyclization on cleavage from resin (strategy 2); and solid phase synthesis and subsequent cleavage of linear peptides from resin, followed by liquid phase cyclization (strategy 3). The cyclic peptide precursors were finally converted to the corresponding CHAPs by side chain modification to the hydroxamic acid. The details of the procedures and experimental data concerning the synthesis of compounds **3-6** are described in the supplemental text (which is published as supplemental data on the PNAS web site, www.pnas.org).

Preparation of Recombinant HDACs and Assay for Enzyme Activity. Cells ($1–2 \times 10^6$, NIH 3T3 for HDAC1, and 293 for HDAC4 and HDAC6) were grown in a 100-mm dish for 24 h, and transfected transiently with 10 μg of each vector, pcDNA3-HD1 for human HDAC1 (27), pcDNA3.1(+)-HD4 for human HDAC4 (28), or pcDNA-mHDA2/HDAC6 for mouse HDAC6 (17), using the LipofectAmine reagent (Life Technologies, Rockville, MD). After successive cultivation in OPTI-MEM for 5 h and DMEM for 19 h, the cells were washed with PBS and lysed by sonication in lysis buffer containing 50 mM Tris·HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40. The soluble fraction collected by microcentrifugation was precleared by incubating with Protein A/G plus agarose beads (Santa Cruz Biotechnology). After the cleared supernatant had been incubated for 1 h at 4°C with 8 μg/ml of an anti-FLAG M2 antibody (Sigma) for HDAC1 and HDAC4, or an anti-HA antibody (Santa Cruz Biotechnology) for HDAC6, the agarose beads were washed three times with lysis buffer and once with HD buffer consisting of 20 mM Tris·HCl (pH 8.0), 150 mM NaCl, 10% glycerol, and a complete protease inhibitor mixture (Boehringer Mannheim). The bound proteins were released from the immune complex by incubating for 1 h at 4°C with 100 μg of the FLAG or the HA peptide (Sigma-Aldrich) in HD buffer (1.25 ml). The supernatant was collected after centrifugation and diluted to give 1,000–2,000 cpm in the following standard enzyme assay. The beads-bound HDAC1 and HDAC6 were used for the drug reversibility test. For the enzyme assay, 10 μl of [^{3}H]acetyl-labeled histones (25,000 cpm/10 μg) was added to 90 μl of the enzyme fraction, and the mixture was incubated at 37°C for 15 min. The enzyme reaction was linear for at least 1 h under these conditions. The reaction was stopped by the addition of 10 μl of concentrated HCl. The released [^{3}H]acetic acid was extracted with 1 ml of ethylacetate, and 0.9 ml of the solvent layer was taken into 5 ml of ACS (aqueous counting scintillant) II solution (Amersham Pharmacia) for determination of radioactivity. The 50% inhibitory concentrations (IC_{50}) were determined as the means ± SD of the concentrations calculated from at least three independent dose–response curves.

Extraction of Histones and Acid/Urea/Triton Gel Electrophoresis. Histones of cultured cells were extracted as described previously (29). The level of histone acetylation was analyzed by slab gel electrophoresis using an acid/urea/Triton (AUT) gel (1 M acetic acid, 8 M urea, 0.5% Triton X-100, 45 mM NH3, and 16% acrylamide) with an upper gel (1 M acetic acid, 6.3 M urea, and 4.4% acrylamide). After the extracted histones had been mixed with loading buffer (7.4 M urea, 1.4 M NH3, 10 mM DTT), electrophoresis was performed in 0.2 M glycine and 1 M acetic acid, and then the gels were stained with silver.

Western Blot Analysis. Lysates from HeLa cells treated with various concentrations of TSA and CHAPs for 24 h were prepared by lysing cells with brief sonication in IP buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 0.1 mM PMSF, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4, 10 μg/ml aprotinin, and 10 mM leupeptin, pH 7.5). Lysates were centrifuged for 10 min at 4°C, and the supernatants were frozen until analysis. An equal amount of proteins (30 μg protein/lane) was loaded and electrophoresed on SDS/polyacrylamide gels, and proteins separated were transferred onto an Immobilon-P membrane (Millipore). An anti-human Cyclin A antibody (Santa Cruz Biotechnology), an anti-human Cyclin D1 antibody (Santa Cruz Biotechnology), an anti-human Cyclin E antibody (Upstate Biotechnology, Lake Placid, NY), an anti-human $p21^{WAF1/Cip1}$ antibody (Transduction Laboratories, Lexington, KY) were used, and the immune complexes were detected with an ECL Western blotting kit (Amersham Pharmacia).

MHC Class-I Molecule Up-Regulation Assay. The activity of HDAC inhibitors to induce expression of MHC class-I molecules was

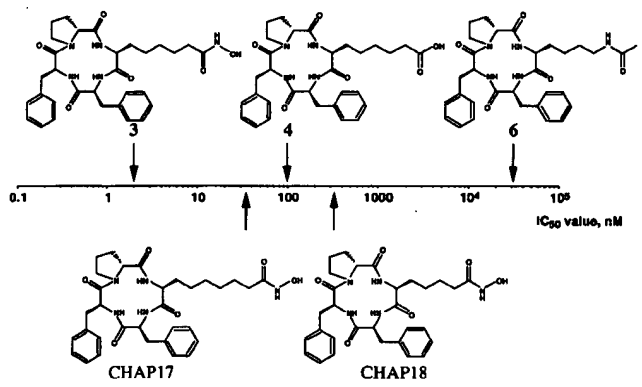

Fig. 2. Effects of the structures of the functional group and aliphatic chain length on the HDAC1-inhibitory potency of CHAP1. The half-maximal inhibitory concentrations (IC_{50}) of CHAP1 (**3**), cyclo(Asu-Phe-Phe-D-Pro) (**4**), cyclo(Lys(Ac)-Phe-Phe-D-Pro) (**6**), cyclo(Aaz(NHOH)-Phe-Phe-D-Pro)(Aaz = α-aminoazelaic acid) (CHAP17), and cyclo(Api(NHOH)-Phe-Phe-d-Pro) (Api = α-aminopimelic acid) (CHAP18) were plotted. The IC_{50} of cyclo(Lys-Phe-Phe-D-Pro) (**5**) was over 100 μM.

evaluated by determining the concentration for 2-fold up-regulation ($C_{\times 2}$) according to the method reported previously (30). Briefly, after 24 h of culture, B16/BL6 cells (5,000 cells/200 μl) in a 96-well microplate were incubated with various concentrations of drugs for 3 days. The expression of MHC class I molecule on the cell surface was measured by a cell ELISA method, and their $C_{\times 2}$ values were calculated from at least three independent dose-response curves.

Flow Cytometry. Procedures for preparation and staining of nuclear DNA with propidium iodide in HeLa cells were described previously (31).

Results

CHAP1 Is a Potent and Reversible Inhibitor of HDAC1. To examine the role of the epoxyketone group of TPX B [**1**, cyclo(Aoe-Phe-Phe-D-Pro)] in HDAC inhibition, we synthesized a series of cyclic tetrapeptide compounds containing L-Asu(NHOH) (**3**), L-aminosuberic acid (**4**), L-lysine (**5**), or acetylated L-lysine (**6**) instead of Aoe, and examined their ability to inhibit HDAC1. Compound **3** was found to be a strong inhibitor, blocking the enzyme activity at an IC_{50} of about 1.9 nM (Fig. 2). To our knowledge, there have been no other reports of a synthetic HDAC inhibitor stronger than TSA (Table 1). We named compound **3** CHAP1. On the other hand, compound **5** containing a free amino group was almost inactive ($IC_{50} > 100$ μM, data not shown). These results indicate that not only the epoxyketone but also the hydroxamic acid acts as a potent functional group for HDAC inhibition in the cyclic tetrapeptide antibiotic. Interestingly, compound **4** containing a carboxylic acid group showed a significant activity ($IC_{50} = 100 \pm 28$ nM), whereas compound **6** containing an acetylated lysine was very weak ($IC_{50} = 31{,}000 \pm 4{,}400$ nM).

We next compared the activity of CHAP compounds having different methylene chain lengths (4, 5, and 6) between their hydroxamic acid and the cyclic tetrapeptide core. As shown in Fig. 2, CHAP with the 5-carbon-long chain (**3**) was much more effective than 6-carbon (CHAP17) and 4-carbon (CHAP18) chains. Because the aliphatic chain length of 5 corresponds to the length between the carbonyl group and the α-carbon in acetylated lysine, it is reasonable to assume that the hydroxamic acid-containing side chain of CHAPs with a 5-carbon-long chain just fits the substrate's pocket of HDAC with which acetylated lysine interacts (Fig. 1*B*).

We previously reported that the inhibition of HDAC by TPX was apparently irreversible (26). This irreversible inhibition is probably due to the alkylation of the enzyme through its epoxy group, because the reduction of the epoxide caused almost complete loss of HDAC-inhibitory activity of TPX. On the other hand, TSA was a reversible inhibitor (29). To test whether the effect of CHAP1 is reversible or not, we incubated HDAC1 immobilized on the agarose beads with CHAP1, TSA, or TPX for 20 min, washed the beads thoroughly, and then analyzed the enzyme activity associated with the beads-conjugated HDAC1 in the absence of the inhibitors. As shown in Fig. 3, the activity of the enzyme that had been treated with CHAP1 was recovered to almost the initial level, whereas that of the enzyme treated with TPX B was not. These results clearly demonstrated that the replacement of the epoxyketone group with the hydroxamic acid converted the inhibitor to a reversible one.

Different Sensitivity of HDAC1 and HDAC6 to CHAP1. Although a number of natural and synthetic compounds have been described as HDAC inhibitors, little is known about their target enzyme specificity. Class I deacetylases related to yeast Rpd3 includes HDAC1 (7), HDAC2 (12), HDAC3 (13), and HDAC8 (14), whereas class II related to Hda1 contains HDAC4 (15, 16), HDAC5 (16, 17), HDAC6 (16, 17), and HDAC7 (18). It has been reported that HDAC1 and HDAC2 are found in the same complex (12), whereas HDAC4 and HDAC5 are associated with HDAC3 (16). HDAC6, a particular isoform that contains two

Table 1. HDAC inhibitory activity and MHC-inducing activity of CHAPs and related compounds

Compound	Structure	Type*	$IC_{50} \pm$ SD, nM				$C_{x2} \pm$ SD, nM
			HDAC1	HDAC4	HDAC6	HDAC6/HDAC1	MHC
TSA			6.0 ± 2.5	38 ± 4	8.6 ± 1.4	1.4	2.8 ± 2.0
TPX A	cyclo(Aoe-Phe-Phe-D-Pip)		0.82 ± 0.29	NT†	524 ± 240	640	3.5 ± 0.6
TPX B	cyclo(Aoe-Phe-Phe-D-Pro)		0.11 ± 0.01	0.30 ± 0.03	360 ± 160	3,300	1.2 ± 0.9
Chlamydocin	cyclo(Aoe-Aib-Phe-D-Pro)		0.15 ± 0.03	NT	1,100 ± 430	7,300	4.6 ± 2.7
Cyl-2	cyclo(Aoe-D-Tyr(Me)-Ile-Pip)		0.70 ± 0.45	NT	40,000 ± 11,000	57,000	10 ± 7
CHAP56	cyclo(Asu(NHOH)-Phe-Phe-D-Pip)	TPX A	6.1 ± 1.4	NT	150 ± 84	25	30 ± 5
CHAP1	cyclo(Asu(NHOH)-Phe-Phe-D-Pro)	TPX B	1.9 ± 0.5	2.7 ± 1.3	19 ± 3	10	98 ± 23
CHAP49	cyclo(Asu(NHOH)-D-Tyr(Me)-Ile-Pip)	Cyl-2	1.2 ± 0.7	NT	36 ± 17	30	5.3 ± 2.2
CHAP30	cyclo(Asu(NHOH)-D-Tyr(Me)-Ile-Pro)	Cyl-1	4.4 ± 1.8	NT	110 ± 84	25	17 ± 8
CHAP53	cyclo(Asu(NHOH)-D-Phe-Leu-Pip)	WF3161	0.94 ± 0.33	NT	22 ± 10	23	20 ± 4
CHAP15	cyclo(Asu(NHOH)-Aib-Phe-D-Pro)	Chlamydocin	0.44 ± 0.23	NT	38 ± 12	86	33 ± 9
CHAP13	cyclo(Asu(NHOH)-D-Pro-Ala-D-Ala)	HC-toxin	2.9 ± 2.1	NT	61 ± 14	21	410 ± 86

*Type, the natural product having the same cyclic tetrapeptide core structure as that of each CHAP.
†NT, Not tested; Aib, α-aminoisobutylic acid.

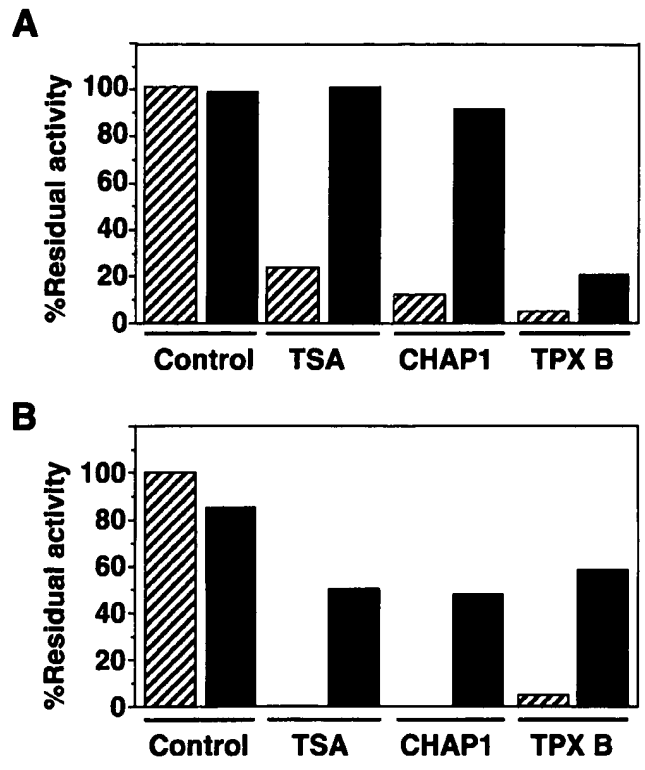

Fig. 3. Reversibility of HDAC inhibition. (*A*) Effects on HDAC1. Beads-conjugated, affinity-purified HDAC1 was pretreated with 0.01% dimethyl sulfoxide (control), 100 nM TSA, 100 nM CHAP1, and 100 nM TPX B for 20 min. (*B*) Effects on HDAC6. Beads-conjugated, affinity-purified HDAC6 was pretreated with 0.01% dimethyl sulfoxide (control), 3 μM TSA, 3 μM CHAP1, and 100 μM TPX B for 60 min. The treated enzyme preparations were washed with drug-free HD buffer, and the residual enzyme activity was determined. The hatched and filled bars represent before and after removal of drugs, respectively.

deacetylase domains, has not been described to associate with other known HDACs. We chose HDAC1 as a class I enzyme and HDAC4 and HDAC6 as class II deacetylases, and compared the effects of these inhibitors (Table 1). TSA inhibited all of the deacetylases tested to a similar extent, although HDAC4 was slightly resistant when compared with HDAC1 and HDAC6. TPX also strongly inhibited the enzyme activity of HDAC1 and HDAC4 at subnanomolar concentrations. Surprisingly, however, HDAC6 was highly resistant to TPX. A similar but smaller extent of resistance was also observed with CHAP1 for HDAC6. HDAC1 was most sensitive to CHAP1 among the HDACs tested, and the IC_{50} value for HDAC6 was ten times larger than that for HDAC1 (Table 1). These results suggest that the cyclic tetrapeptide structure is responsible, at least in part, for the weak inhibition of HDAC6 by TPX.

HDAC Inhibition by CHAPs Corresponding to Naturally Occurring Cyclic Tetrapeptide Antibiotics. Chlamydocin, HC-toxin, Cyl-1, Cyl-2, and WF-3161 in addition to TPX A and B have been reported as cyclic tetrapeptide antibiotics containing Aoe (19). Chlamydocin and WF-3161 were described to have antifungal and antitumor activities (32, 33). HC-toxin, Cyl-1, and Cyl-2 are phytotoxic substances produced by phytopathogenic fungi, causing necrotic lesions on the leaves and sometimes serious reduction of crop yield (34–36). Of these compounds, HC-toxin and chlamydocin have been described to inhibit HDAC (20). We next synthesized CHAPs corresponding to all these naturally occurring cyclic tetrapeptide antibiotics, and their IC_{50} values for HDAC1 and HDAC6 were determined (Table 1). All CHAPs synthesized were potent inhibitors of HDAC1, effective at low nanomolar concentrations or subnanomolar concentrations. CHAP15 (chlamydocin-type) was the most potent inhibitor (IC_{50} = 0.44 nM). Again, HDAC6 was more than 10-fold resistant to these CHAPs. The difference in the IC_{50} between HDAC1 and HDAC6 varied with the structure of the cyclic tetrapeptide. In particular, the activity of CHAP15 to inhibit HDAC1 was 87-fold stronger than that needed to inhibit HDAC6. Interestingly, natural products containing Aoe (TPX A, TPX B, chlamydocin, and Cyl-2) showed strikingly large IC_{50} values for HDAC6 (360–40,000 nM), compared with those at subnanomolar levels for HDAC1 (Table 1). These results suggest that the epoxyketone group enhances the selectivity to inhibit HDAC1.

Reversible Inhibition of HDAC6 by TPX. Because TPX has been shown to be a slow binding inhibitor (26), we tested whether the prolonged preincubation of HDAC6 with TPX results in the stronger inhibition. The inhibitory effect was almost unchanged after the 10-h incubation (data not shown). We next asked whether HDAC6 is irreversibly inhibited by TPX as was HDAC1. The affinity-purified, beads-conjugated HDAC6 was incubated with the high concentration of TSA (3 μM), CHAP1 (3 μM), or TPX B (100 μM) for 60 min, and then the residual enzyme activity was determined in the presence or absence of the drug (Fig. 3*B*). The activity of HDAC6 that had been treated with TPX B was recovered to levels similar to those with TSA and CHAP1 after removal of the drugs. These results suggest that TPX is a non-alkylating, reversible inhibitor for HDAC6.

CHAPs Inhibit HDAC Activity *in Vivo* and Affect Gene Expression and Cell Cycle Progression. To examine the *in vivo* HDAC inhibition by CHAPs, we treated murine B16/BL6 melanoma cells with various concentrations of TSA and CHAPs, and the effects on histone acetylation were analyzed by AUT gel electrophoresis. AUT gel electrophoresis allows separation of each cellular histone (H1, H2A, H2B, H3, and H4) with the different extent of acetylation, because of slower migration rates of the acetylated species. Fig. 4 shows the profiles of histones H4 and H2B

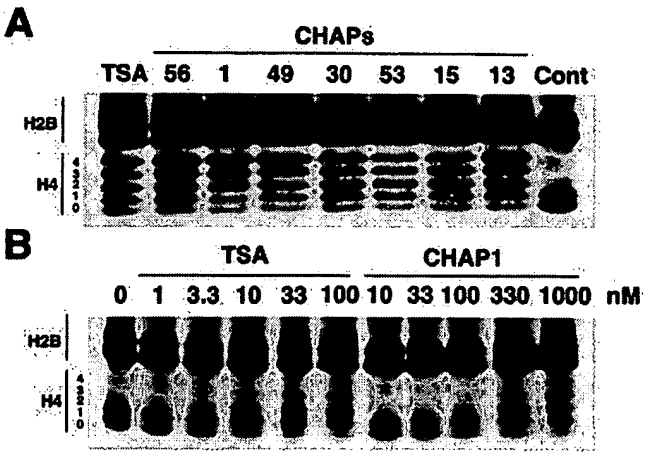

Fig. 4. Effects of CHAPs on histone acetylation *in vivo*. (*A*) Acetylation by TSA and CHAPs. B16/BL6 cells were treated with TSA (1 μM), CHAP56 (1 μM), CHAP1 (10 μM), CHAP49 (1 μM), CHAP30 (1 μM), CHAP53 (1 μM), CHAP15 (1 μM), and CHAP13 (10 μM) for 6 h, and the level of histone acetylation was determined by AUT gel electrophoresis as described in *Materials and Methods*. (*B*) Dose-response. B16/BL6 cells were treated with the indicated concentrations of TSA and CHAP1 for 6 h, and the level of histone acetylation was determined.

extracted from TSA- or CHAP-treated B16/BL6 melanoma cells. All CHAPs as well as TSA apparently induced the accumulation of highly acetylated histones, characterized by decreases in the most rapidly migrating bands of each histone species and appearance of additional slower migrating ones like a ladder on the AUT gel (Fig. 4*A*). These results clearly demonstrate that CHAPs inhibit HDAC not only *in vitro* but also in the cells. The minimal effective concentrations of TSA and CHAP1 for the increased histone acetylation were 3.3 nM and 100 nM, respectively (Fig. 4*B*).

The B16/BL6 melanoma cells are morphologically differentiated and express the MHC class-I molecule when treated with HDAC inhibitors (30). The effective concentrations of TSA and CHAP1 to induce the MHC class-I were 2.8 nM and 98 nM, respectively, which almost coincided with those required for the increased histone acetylation (Table 1). All CHAPs except CHAP13 (HC-toxin type) could induce the MHC class-I expression at the concentrations below 100 nM. The relatively weak *in vivo* activity of CHAP13 may be ascribed to low membrane permeability because of lack of hydrophobic aromatic amino acid residues in the cyclic tetrapeptide core.

We finally analyzed the effects of CHAP compounds on the cell cycle and the expression of G_1 cyclins and p21 in HeLa cells. Indeed, a marked increase in p21 expression and a decrease in cyclin A have previously been observed during the cell cycle arrest after HDAC inhibitor treatment (37–40). As shown in Fig. 5*A*, flow cytometric analysis showed that CHAP1 treatment caused an increase in the G_1 cell population and a decrease in the S-phase cells at the concentration of 0.1 μM. Moreover, an arrest at both G_1 and G_2 phases was observed at the concentration of 1 μM or higher. TSA also induced an arrest of the cell cycle, but higher concentrations of the inhibitor were required to induce the cell cycle arrest in HeLa cells as described (40). Analysis of the cell cycle proteins showed that all CHAPs tested caused a down-regulation of cyclin A and a drastic induction of p21 in HeLa cells at the concentration of 0.1 μM, whereas expression of cyclin D1 and cyclin E was essentially unchanged (Fig. 5*B*). These effects were almost the same as those induced by TSA treatment, although the induction of p21 required 1 μM of TSA in this particular experiment, probably because of a partial degradation or inactivation of TSA in HeLa cells.

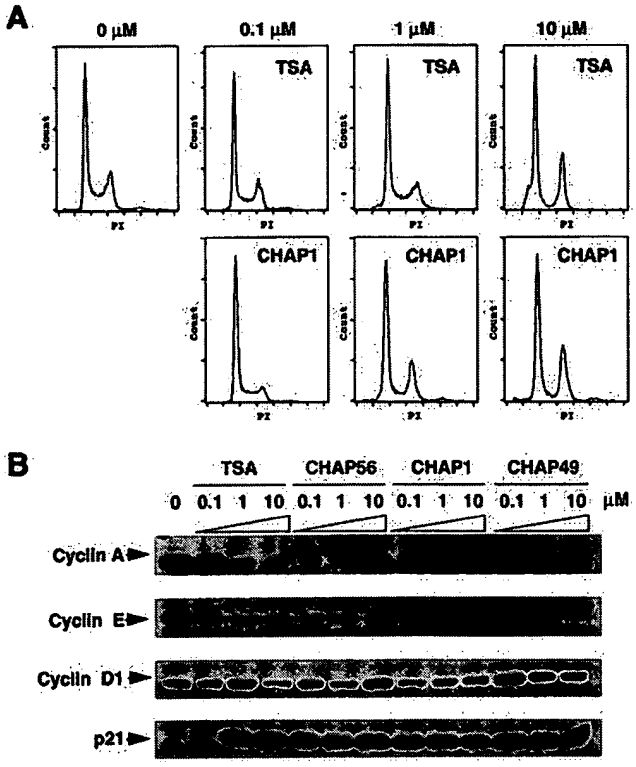

Fig. 5. Effects of CHAPs on the cell cycle and expression of cell cycle proteins. (*A*) HeLa cells were treated with various concentrations of TSA and CHAP1 for 24 h, and the isolated nuclei stained with propidium iodide (PI) were analyzed by flow cytometry. (*B*) HeLa cells were treated with the indicated concentrations of TSA, CHAP56, CHAP1, and CHAP49 for 24 h, and the amounts of cyclin A, cyclin E, cyclin D1, and p21Waf1 were determined by Western blotting.

Discussion

We showed that a series of synthetic compounds that were built from TSA and TPX-like cyclic tetrapeptide antibiotics reversibly inhibited HDAC1 at low nanomolar or subnanomolar concentrations. Crystallographic studies using the HDLP–TSA complex have shown that TSA binds by inserting its long aliphatic chain into the tube-like HDLP pocket and inhibits the enzyme activity by interacting with the zinc and active-site residues through its hydroxamic acid at one end of the aliphatic chain (24). The importance of the hydroxamic acid and the length of the aliphatic chain of CHAP1 in inhibiting HDAC1 support the idea that the side chain of Aoe in TPX is also a substrate mimic. Although the naturally occurring cyclic tetrapeptide antibiotics containing Aoe are potent in inhibiting HDAC *in vitro*, they show only very weak activity in the animal models (41). This weak activity is probably due to the chemical instability of the epoxyketone group in blood. CHAPs may circumvent this problem in the drug development because of the absence of the epoxyketone. In fact, the half-life of a CHAP compound {cyclo[Asu(NHOH)-D-Tyr(Me)-Ile-D-Pro]} in rat blood was about 50 min (unpublished results), whereas that of chlamydocin is 2.5 min (41).

Short-chain fatty acids such as *n*-butyrate are well known to inhibit HDAC. However, milimolar concentrations are required for inducing histone hyperacetylation *in vivo* or inhibiting HDAC *in vitro* (42). We showed that a TPX B analogue containing a carboxylic acid instead of the epoxyketone group (**4**) inhibited HDAC1 at the concentration of about 100 nM. Although this inhibitor is much weaker than CHAP1 (**3**), it is the most active carboxylic acid-containing inhibitor known to date. This finding indicates that the cyclic tetrapeptide structure confers the high affinity with HDAC1 onto the short-chain fatty acids, thereby potentiating the inhibitory activity. This high affinity is consistent with the model that the cyclic tetrapeptide with hydrophobic groups serves as a cap necessary for packing the inhibitor at the rim of the tube-like active-site pocket (24).

Accumulating data have suggested that each member of the HDAC family is a component of a distinct physical complex playing a distinct role in gene expression. For example, a large protein complex containing HDAC1 binds the E2F transcription factor via association with the retinoblastoma tumor suppressor protein (43, 44), whereas HDAC4 and HDAC5 associate specifically with the myocyte enhancer factor MEF2A and repress MEF2A-dependent transcription (45, 46). It is therefore likely that inhibition of a specific isoform leads to changes in transcription of a specific subset of genes. However, such an isoform-specific inhibitor has not yet been developed. TSA and suberoylanilide hydroxamic acid, simple analogues of acetylated lysine with small cap groups, may cause nonselective inhibition of class I and II HDACs. On the other hand, the cyclic tetrapeptide structure, which probably makes extensive contacts at the rim of the pocket and in the shallow grooves surrounding the pocket entrance, may mimic the substrate structure surrounding acetylated lysine (Fig. 1*B*). If so, it would be likely that the target enzyme specificity of CHAP can be modulated by changing amino acids in the cyclic tetrapeptide. In this study, we showed that CHAPs, as well as several cyclic tetrapeptide antibiotics

containing Aoe, were less active against HDAC6 than HDAC1, supporting the idea that the cyclic tetrapeptide structure is responsible for the target enzyme specificity. HDAC6 is a unique isoform in which the catalytic domain is internally duplicated (16, 17). Furthermore, it is normally localized in the cytoplasm, and only a fraction of the protein relocalizes into the nucleus in response to stimuli for differentiation, suggesting that its natural substrates include non-histone acetylated proteins (47). The cyclic tetrapeptide structures of CHAPs synthesized in this study may be distant from the natural substrates for HDAC6.

The reversibility test showed that the effect of TPX on HDAC6, but not on HDAC1, was reversible (Fig. 3), suggesting that TPX does not alkylate HDAC6. This inability of TPX to alkylate HDAC6 is probably responsible for the higher resistance of HDAC6 to the Aoe-containing cyclic tetrapeptide antibiotics than to CHAPs (Table 1). Because the epoxide may be required for the alkylation of a catalytic pocket residue of HDAC1, presumably one of the conserved charge-relay histidines (Fig. 1*B*; ref. 26), it seems possible that a subtle difference in the position of the target residue results in the absence of the crosslink to HDAC6. The IC_{50} of these compounds for HDAC6 inhibition ranging between 100 nM and 100 μM were consistent with those for HDAC1 of non-chelating derivatives of CHAP1 such as compounds **4** and **6** (Fig. 2).

In summary, we showed potent and specific inhibition of HDAC1 by CHAPs. Our data suggest that the cyclic tetrapeptide portion affects both enzyme inhibitory potency and specificity, whereas the hydroxamic acid group acts as a potent enzyme-inhibiting group with *in vivo* stability better than the epoxy-ketone. These observations raise the possibility of developing the isoform-specific inhibitors based on CHAP. Because a marked structural diversity can be obtained by combinatorial synthesis of the cyclic tetrapeptide, CHAP is a promising framework to develop isoform-specific HDAC inhibitors. These specific HDAC inhibitors are clearly important for not only elucidating a downstream signaling step of each member of HDAC but also improving their therapeutical potential for transcription therapy and chemotherapy.

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1. Grunstein, M. (1997) *Nature (London)* **389,** 349–352.
2. Struhl, K. (1998) *Genes Dev.* **12,** 599–606.
3. Kouzarides, T. (1999) *Curr. Opin. Genet. Dev.* **9,** 40–48.
4. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. & Allis, C. D. (1996) *Cell* **84,** 843–851.
5. Ogryzko, V. V., Schitz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. (1996) *Cell* **87,** 953–959.
6. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. & Nakatani, Y. (1996) *Nature (London)* **382,** 319–324.
7. Taunton, J., Hassig, C. A. & Schreiber, S. L. (1996) *Science* **272,** 408–411.
8. Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M. & Grunstein, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14503–14508.
9. Imai, S., Armstrong, C. M., Kaeberlein, M. & Guarente, L. (2000) *Nature (London)* **403,** 795–800.
10. Kuo, M. H. & Allis, C. D. (1998) *BioEssays* **20,** 615–626.
11. Torchia, J., Glass, C. & Rosenfeld, M. G. (1998) *Curr. Opin. Cell Biol.* **10,** 373–383.
12. Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E. & Eisenman, R. N. (1997) *Cell* **89,** 349–356.
13. Emiliani, S., Fischle, W., Van, L. C., Al, A. Y. & Verdin, E. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2795–2800.
14. Hu, E., Chen, Z., Fredrickson, T., Zhu, Y., Kirkpatrick, R., Zhang, G. F., Johanson, K., Sung, C. M., Liu, R. & Winkler, J. (2000) *J. Biol. Chem.* **275,** 15254–15264.
15. Wang, A. H., Bertos, N. R., Vezmar, M., Pelletier, N., Crosato, M., Heng, H. H., Th'ng, J., Han, J. & Yang, X. J. (1999) *Mol. Cell. Biol.* **19,** 7816–7827.
16. Grozinger, C. M., Hassig, C. A. & Schreiber, S. L. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 4868–4873.
17. Verdel, A. & Khochbin, S. (1999) *J. Biol. Chem.* **274,** 2440–2445.
18. Kao, H. Y., Downes, M., Ordentlich, P. & Evans, R. M. (2000) *Genes Dev.* **14,** 55–66.
19. Yoshida, M., Horinouchi, S. & Beppu, T. (1995) *BioEssays* **17,** 423–430.
20. Brosch, G., Ransom, R., Lechner, T., Walton, J. D. & Loidl, P. (1995) *Plant Cell* **7,** 1941–1950.
21. Marks, P. A., Richon, V. M. & Rifkind, R. A. (2000) *J. Natl. Cancer Inst.* **92,** 1210–1216.
22. Nakajima, H., Kim, Y. B., Terano, H., Yoshida, M. & Horinouchi, S. (1998) *Exp. Cell Res.* **241,** 126–133.
23. Saito, A., Yamashita, T., Mariko, Y., Nosaka, Y., Tsuchiya, K., Ando, T., Suzuki, T., Tsuruo, T. & Nakanishi, O. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 4592–4597.
24. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R. & Pavletich, N. P. (1999) *Nature (London)* **401,** 188–193.
25. Richon, V. M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R. A. & Marks, P. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3003–3007.
26. Kijima, M., Yoshida, M., Sugita, K., Horinouchi, S. & Beppu, T. (1993) *J. Biol. Chem.* **268,** 22429–22435.
27. Yang, W. M., Yao, Y. L., Sun, J. M., Davie, J. R. & Seto, E. (1997) *J. Biol. Chem.* **272,** 28001–28007.
28. Fischle, W., Emiliani, S., Hendzel, M. J., Nagase, T., Nomura, N., Voelter, W. & Verdin, E. (1999) *J. Biol. Chem.* **274,** 11713–11720.
29. Yoshida, M., Kijima, M., Akita, M. & Beppu, T. (1990) *J. Biol. Chem.* **265,** 17174–17179.
30. Komatsu, Y. & Hayashi, H. (1998) *J. Antibiot.* **51,** 89–91.
31. Yoshida, M. & Beppu, T. (1988) *Exp. Cell Res.* **177,** 122–131.
32. Closse, A. & Hugenin, R. (1974) *Helv. Chim. Acta* **57,** 533–545.
33. Umehara, K., Nakahara, K., Kiyoto, S., Iwami, M., Okamoto, M., Tanaka, H., Kohsaka, M., Aoki, H. & Imanaka, H. (1983) *J. Antibiot.* **36,** 478–483.
34. Liesch, J. M., Sweeley, C. C., Staffeld, G. D., Anderson, M. S., Weber, D. J. & Scheffer, R. P. (1982) *Tetrahedron* **38,** 45–48.
35. Hirota, A., Suzuki, A., Aizawa, K. & Tamura, S. (1973) *Agric. Biol. Chem.* **37,** 955–956.
36. Takayama, S., Isogai, A., Nakata, M., Suzuki, H. & Suzuki, A. (1984) *Agric. Biol. Chem.* **48,** 839–842.
37. Archer, S. Y., Meng, S., Shei, A. & Hodin, R. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6791–6796.
38. Vaziri, C., Stice, L. & Faller, D. V. (1998) *Cell Growth Differ.* **9,** 465–474.
39. Kim, Y. B., Lee, K.-H., Sugita, K., Yoshida, M. & Horinouchi, S. (1999) *Oncogene* **18,** 2461–2470.
40. Kim, Y. B., Ki, S. W., Yoshida, M. & Horinouchi, S. (2000) *J. Antibiot.* **53,** 1191–1200.
41. Stähelin, H. & Trippmacher, A. (1974) *Eur. J. Cancer* **10,** 801–808.
42. Cousens, L. S., Gallwitz, D. & Alberts, B. M. (1979) *J. Biol. Chem.* **254,** 1716–1723.
43. Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J. & Kouzarides, T. (1998) *Nature (London)* **391,** 597–601.
44. Magnaghi, J. L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le, V. J., Troalen, F., Trouche, D. & Harel, B. A. (1998) *Nature (London)* **391,** 601–605.
45. Miska, E. A., Karlsson, C., Langley, E., Nielsen, S. J., Pines, J. & Kouzarides, T. (1999) *EMBO J.* **18,** 5099–5107.
46. Lemercier, C., Verdel, A., Galloo, B., Curtet, S., Brocard, M. P. & Khochbin, S. (2000) *J. Biol. Chem.* **275,** 15594–15599.
47. Verdel, A., Curtet, S., Brocard, M. P., Rousseaux, S., Lemercier, C., Yoshida, M. & Khochbin, S. (2000) *Curr. Biol.* **10,** 747–749.

Exhibit 2

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Medical Sciences

A synthetic inhibitor of histone deacetylase, MS-27-275, with marked *in vivo* antitumor activity against human tumors

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ABSTRACT **Synthetic benzamide derivatives were investigated for their ability to inhibit histone deacetylase (HDA). In this study, one of the most active benzamide derivatives, MS-27-275, was examined with regard to its biological properties and antitumor efficacy. MS-27-275 inhibited partially purified human HDA and caused hyperacetylation of nuclear histones in various tumor cell lines. It behaved in a manner similar to other HDA inhibitors, such as sodium butyrate and trichostatin A; MS-27-275 induced $p21^{WAF1/CIP1}$ and gelsolin and changed the cell cycle distribution, decrease of S-phase cells, and increase of G_1-phase cells. The *in vitro* sensitivity spectrum of MS-27-275 against various human tumor cell lines showed a pattern different than that of a commonly used antitumor agent, 5-fluorouracil, and, of interest, the accumulation of $p21^{WAF1/CIP1}$ tended to be faster and greater in the cell lines sensitive to MS-27-275. MS-27-275 administered orally strongly inhibited the growth in seven of eight tumor lines implanted into nude mice, although most of these did not respond to 5-fluorouracil. A structurally analogous compound to MS-27-275 without HDA-inhibiting activity showed neither the biological effects in cell culture nor the *in vivo* therapeutic efficacy. These results suggest that MS-27-275 acts as an antitumor agent through HDA inhibition and may provide a novel chemotherapeutic strategy for cancers insensitive to traditional antitumor agents.**

Acetylation of nuclear histones, which is regulated by acetyltransferase and deacetylase (1–4), has been supposed to play a crucial role in gene expression because transcriptionally activated genes have been found to be associated with highly acetylated loci whereas transcriptionally inactive genes have been found to be associated with hypoacetylation (5–7). Furthermore, recent molecular and genetic approaches identified histone acetyltransferases and histone deacetylases (HDA) as transcriptional coactivators and transcriptional corepressors, respectively. These observations provide a molecular basis for regulation of transcription through acetylation of histones (8–10).

Although the precise mechanism underlying cell cycle arrest or differentiation through histone acetylation has not been elucidated, sodium n-butyrate (NaBu), an HDA inhibitor (11), has been known to arrest the cell cycle and provide various differentiation phenotypes or revertant phenotypes to cancer cells, including leukemias (12, 13), colorectal cancers (14, 15), a hepatic cancer (16), breast cancers (17, 18), and fibroblasts transformed by a oncogene (19). Therefore, compounds possessing HDA-inhibiting activity have been thought to represent a novel class of agent with less toxicity, along with all-*trans*-retinoic acid (20), for treatment of human cancers.

Although several efforts to apply NaBu for clinical treatment have been reported (21, 22), the efficacies were very limited because of its low antiproliferative activity and short half life in blood. Therefore, several derivatives of NaBu have been studied to improve the rapid metabolism in the body (23–25). Recently, other classes of compounds such as trichostatin A, trapoxin, and depudesin, which are derived from natural products, were reported to exhibit strong HDA inhibition (26–28). Although these natural compounds display strong *in vitro* activity, no *in vivo* antitumor efficacy has been reported, presumably because of instability, low retention, or nonspecific toxicity of the compounds in the body. During our efforts to find novel agents to treat refractory malignancies, including multidrug resistance (29–31), we found a series of synthetic benzamide derivatives with HDA-inhibitory activity *in vitro* and *in vivo* (32). Here, we report the characteristic features of one of these compounds and its strong antitumor efficacy against human cancers in nude mice.

MATERIALS AND METHODS

Chemicals. *N*-(2-aminophenyl)-4-[*N*-(pyridin-3-yl-methoxycarbonyl)aminomethyl]benzamide (MS-27-275, Fig. 1*A*, compound 1) and its 3′-amino derivative (Fig. 1*A*, compound 2) were prepared as described (32).

Cells, Animals, and Antibodies. Human leukemia cell lines K562 and HL-60, human colorectal cancer lines COLO320DM, HT-29, and HCT-15, a human lung cancer line, Calu-3, a human ovary cancer line, SK-OV-3, and a human pancreatic cancer line, Capan-1, were obtained from the American Type Culture Collection and were maintained under the recommended conditions. A2780, a human ovary cancer line, provided by R. Ozols and T. Hamilton (National Cancer Institute), KB-3-1, a human oral cancer line obtained from I. Pastan (National Cancer Institute), and 4-1St and St-4, human gastric cancer lines established in our laboratory, were maintained as described (31). Female BALB/c-nu/nu nude mice (5 or 6 weeks old) were purchased from Charles River Breeding Laboratories. The mice were used at 7 weeks of age. Antibodies specific to p53 and $p21^{WAF1/CIP1}$ were purchased from Santa Cruz Biotechnology, and retinoblastoma protein (pRb) and gelsolin were from QED Bioscience (San Diego, CA) and Sigma (St. Louis, MO), respectively.

Assay for Histone Deacetylase. HDA was partially purified as described by Yoshida *et al.* (26) with slight modifications. K562 cells (2.5×10^8) were disrupted in 15 ml of HDA buffer (15 mM potassium phosphate, pH 7.5/5% glycerol/0.2 mM EDTA). Nuclei of the cells were collected by centrifugation ($35,000 \times g$, 10 min) and were resuspended in 15 ml of HDA buffer containing 1 M $(NH_4)_2SO_4$. After sonication to reduce viscosity, the supernatant was collected by centrifugation, solid

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Abbreviations: HDA, histone deacetylase; pRb, retinoblastoma protein; 5-FU, 5-fluorouracil.

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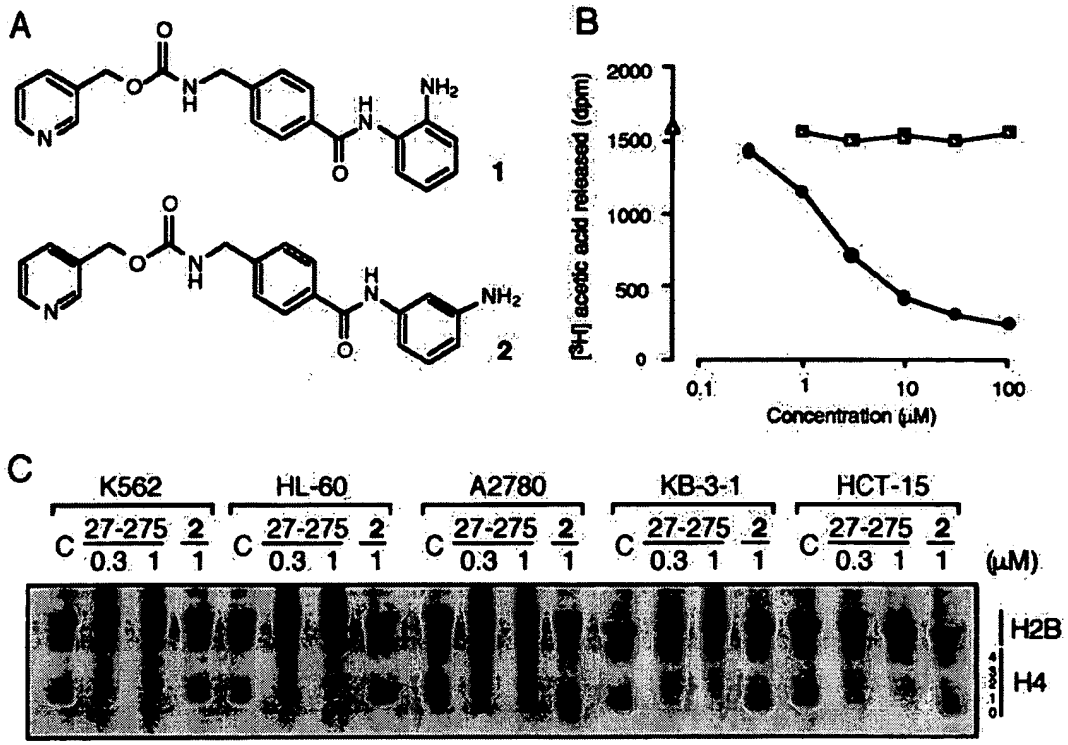

FIG. 1. Effect of MS-27-275 on HDA activity. (*A*) Chemical structure of MS-27-275 (1) and 3'-amino derivative (2). (*B*) Inhibition of human HDA by MS-27-275. HDA activity was measured either in the presence of MS-27-275 (●) or compound 2 (▪) or in the absence of the agent (△). (*C*) Hyperacetylation of nuclear histone (H4) by MS-27-275. Histones (40 μg) extracted from cells exposed to 0.3 μM or 1 μM MS-27-275 or 1 μM compound 2 for 24 h were separated by acid/urea/Triton X-100 gel electrophoresis. The details are described in *Materials and Methods*.

$(NH_4)_2SO_4$ was added to the supernatant to make the final concentration 3.5 M, and was stirred for 1 h at 0°C. The precipitates collected by centrifugation were dissolved again with 4 ml of HDA buffer and were dialyzed against 2 liters of HDA buffer. The dialysate was loaded onto MonoQ HR5/5 (Amersham Pharmacia) equilibrated with HDA buffer, and the proteins were eluted with a linear gradient of 0–1 M NaCl in 30 ml of HDA buffer. A single peak of HDA activity was eluted at 0.4 M NaCl, and the fraction was stored at −80°C until use. Nuclear histones were labeled by incubation of K562 cells (10^8 cells) in a 25 ml of growth medium containing 0.5 mCi/ml [^{3}H]sodium acetate (152.8 GBq/mmol; NEN) and 5 mM NaBu at 37°C for 1 h. Histones were extracted as described (26).

HDA-inhibitory activity of the compound was estimated in 50 μl of reaction mixture containing 2 μl of the above HDA fraction, 100 μg/ml of [^{3}H]acetylated histones, and 5 μl of the compound dissolved in HDA buffer at 37°C for 10 min. [^{3}H]acetic acid released by the reaction was extracted with 50 μl of 1M HCl and 0.55 ml of ethyl acetate, and the radioactivity in the solvent layer was measured by liquid-scintillation counting. To assess *in vivo* HDA inhibition, cellular histones were extracted and examined by acid/urea/Triton X-100 PAGE followed by staining with Coomasie brilliant blue R-250, as described (26).

Northern Blot Analysis. Total RNA was isolated by the acid guanidinium isothiocyanate-phenol-chloroform method. The RNA was separated by electrophoresis through 1% (weight/volume) agarose-formaldehyde gels, was transferred onto nylon membranes (Hybond-N+, Amersham Pharmacia), and was hybridized with a digoxigenin-labeled cRNA specific to human $p21^{WAF1/CIP1}$ or gelsolin by using DIG Easy Hyb (Boehringer Mannheim) under manufacturer's instruction. The probes were prepared by reverse transcription by using random hexamers and, after amplification, by PCR using oligonucleotides specific to human $p21^{CIP1/WAF1}$ cDNA (ACTCAGAGGAGGCGCCATGT and TTCCTGTGGGCGGATTAGGG) or human gelsolin cDNA (GGAAGCCCATGATCATCTAC and TGTACCGCTTAGCAGAAGTC). The PCR products were cloned into the pGEM-T plasmid (Promega) and were confirmed by DNA sequencing. Digoxigenin-labeled cRNAs were synthesized by using a DIG RNA labeling kit (Boehringer Mannheim).

Western Blot Analysis. Cells ($2–6 \times 10^6$) were lysed with 0.3 ml of 63 mM Tris·HCl (pH 6.3), 2 mM EDTA, 5% 2-mercaptoethanol, 2.3% SDS, and 5% glycerol. The proteins were separated by SDS/PAGE and were electrophoretically transferred onto nitrocellulose membranes. The blots were probed with an antibody specific to each protein and were detected by using the enhanced chemiluminescence method (Amersham).

Flow Cytometric Analysis. Unsynchronized cells were seeded at 10^6 per 100-mm dish and were exposed to the agent for 24 h. After fixing with 70% ethanol and treatment with 0.25 μg/ml RNase, the nuclei were stained with 50 μg/ml propidium iodide, and the relative DNA content was measured by using a fluorescence-activated cell sorter (EPICS ELITE, Coulter).

Evaluation of *in Vitro* Sensitivity. Cancer cells (5×10^3) were seeded into each well of 96-well plates and were cultured with graded concentrations of the drugs for 3 days. The cells were stained with 0.1 mg/ml neutral red for 1 h in a CO_2-incubator, and, after aspiration of the medium, OD_{540} of the neutral red solubilized with 50 μl of ethanol and 150 μl of 0.1 M Na_2HPO_4 was measured. The IC_{50} value was determined by plotting growth inhibition of the cells against the logarithm of the drug concentration.

***In Vivo* Antitumor Activity.** A2780 cells (9×10^6) grown *in vitro* were suspended in PBS and were injected subcutaneously into the flank of nude mouse. For the other tumor lines, KB-3-1, HCT-15, 4-1St, Calu-3, St-4, Capan-1, and HT-29, tumors were passaged several times before starting *in vivo* antitumor testing, and a tumor lump (2–3 mm in diameter) was

transplanted subcutaneously into the flank of a nude mouse by using a trocar needle. Treatment (four or five mice in each experimental group) with the drugs was started after the tumors were confirmed to have grown in the body (tumor size, 20–100 mm^3). MS-27-275 and compound 2, both dissolved with 0.05 N HCl, 0.1% Tween 80, and 5-fluorouracil (5-FU) (Mitsui Pharmaceuticals, Tokyo) and diluted with physiological saline, were administered orally once daily 5 days per week for 4 weeks. Tumor length and width were monitored twice weekly, and tumor volume was calculated as described (31).

RESULTS

Inhibition of Histone Deacetylase by MS-27-275. A series of synthetic benzamide derivatives were investigated for their ability to inhibit HDA, and MS-27-275 (Fig. 1*A*, compound 1) was found to be one of the most active compounds. MS-27-275 inhibited HDA purified from human leukemia cells in a dose-dependent manner, and the IC_{50} value was estimated to be 2.0 μM (Fig. 1*B*) whereas trichostatin A and trapoxin were reported to inhibit HDA at nanomolar concentrations in similar assays (26, 27). The addition of MS-27-275 to cell culture resulted in the accumulation of hyperacetylated H4 molecules that produced multiple bands corresponding to histones with one, two, three, and four acetylated lysine residues on acid/urea/Triton X-100 gels (Fig. 1*C*). The levels of acetylation were almost identical among the cell lines examined (Fig. 1*C*). Of interest, compound 2, a structural analogue of MS-27-275 with a 3′-aminophenyl instead of a 2′-aminophenyl group (Fig. 1*A*), showed no effect on either the activity of HDA (Fig. 1*B*) or on the acetylation state of cellular histones (Fig. 1*C*), suggesting that the 2′-amino group of MS-27-275 plays an important role in binding and inhibition of the enzyme.

Induction of $p21^{WAF1/CIP1}$ and Gelsolin by MS-27-275. Other HDA inhibitors such as NaBu and trichostatin A were reported to transcriptionally induce $p21^{WAF1/CIP1}$ (33, 34) and gelsolin (35, 36), both of which are considered to be tumor suppressors. MS-27-275 also increased the intracellular amounts of $p21^{WAF1/CIP1}$ and gelsolin. In the K562 cells, mRNAs specific to both proteins were identified after 24-h exposure to MS-27-275, and the proteins were clearly accumulated after 48-h exposure (Fig. 2*A*). Compound 2 affected neither mRNAs nor protein expression (Fig. 2*A*).

Because it is well known that $p21^{WAF1/CIP1}$ inhibits cyclin-dependent kinases (37–40) and that the $p21^{WAF1/CIP1}$ gene is physiologically induced by p53 (41), we checked modulation of p53, $p21^{WAF1/CIP1}$, and pRb by MS-27-275 in several tumor lines. Among the tumor lines examined, K562, HL-60, KB-3-1, and HCT-15 cell lines had no intrinsic $p21^{WAF1/CIP1}$ whereas A2780 had small but significant amounts of intrinsic $p21^{WAF1/CIP1}$ (Fig. 2*B*). This observation was consistent with the previous studies that reported that K562, HL-60, and HCT-15 cell lines had dysfunctional alterations in p53 gene (42, 43) and that the A2780 line had wild-type p53 gene (44), except for KB-3-1, whose status of p53 gene has not been reported. After the exposure to MS-27-275, the cells accumulated significant amounts of $p21^{WAF1/CIP1}$ irrespective of the amount of p53 and the status of p53 gene (Fig. 2*B* and Table 1). The induction of $p21^{WAF1/CIP1}$ resulted in the reduction of hyperphosphorylated pRb molecules, which migrated more slowly than the hypophosphorylated form on SDS polyacrylamide gels (46–48) (Fig. 2*B*). Treatment of A2780 cells with MS-27-275 clearly decreased the population of S-phase cells and increased G_1-phase cells (Fig. 3*B*), although compound 2, which showed neither HDA inhibition nor induction of $p21^{WAF1/CIP1}$ or gelsolin, showed no effect on the cell cycle distribution (Fig. 3*C*). These observations suggest that, similarly to the other HDA inhibitors, MS-27-275 transcriptionally induced $p21^{WAF1/CIP1}$ and gelsolin through acetylation of histones and changed the cell cycle distribution.

***In Vitro* Sensitivity and Induction of $p21^{WAF1/CIP1}$ by MS-27-275 in Various Tumor Cells.** *In vitro* antiproliferative activity of MS-27-275 was examined in human tumor cell lines of various origins. The sensitivity spectrum of MS-27-275 against these cell lines, which showed various IC_{50} values ranging from 0.0415 μM (A2780) to 4.71 μM (HCT-15), was different from that of the commonly used chemotherapeutic agent 5-FU (Table 1). The antiproliferative activity of compound 2 was much weaker than that of MS-27-275 in all of the lines tested, suggesting that the antiproliferative activity is mainly caused by the HDA-inhibitory action of the compound but not by its nonspecific toxicity.

Of interest, the accumulation of $p21^{WAF1/CIP1}$ in the cells exposed to MS-27-275 tended to be faster and greater in cell lines sensitive to MS-27-275, such as A2780, Calu-3, HL-60, and K562 (IC_{50} values were 0.0415 μM, 0.195 μM, 0.212 μM, and 0.589 μM, respectively), as compared with the others (Fig. 4*A* and Table 1). On the other hand, induction of gelsolin by MS-27-275 seemed to have no correlation with the sensitivities of the cells (Fig. 4*B* and Table 1), although gelsolin was reported to be a tumor suppressor (49, 50). From these observations, it is possible that the induction of $p21^{WAF1/CIP1}$ through histone acetylation plays one of the crucial roles in the action of MS-27-275. Because the levels of acetylation were almost equal among these cell lines, it is suggested that the variations in the sensitivity to MS-27-275 and the kinetics of the induction of these proteins were derived from different

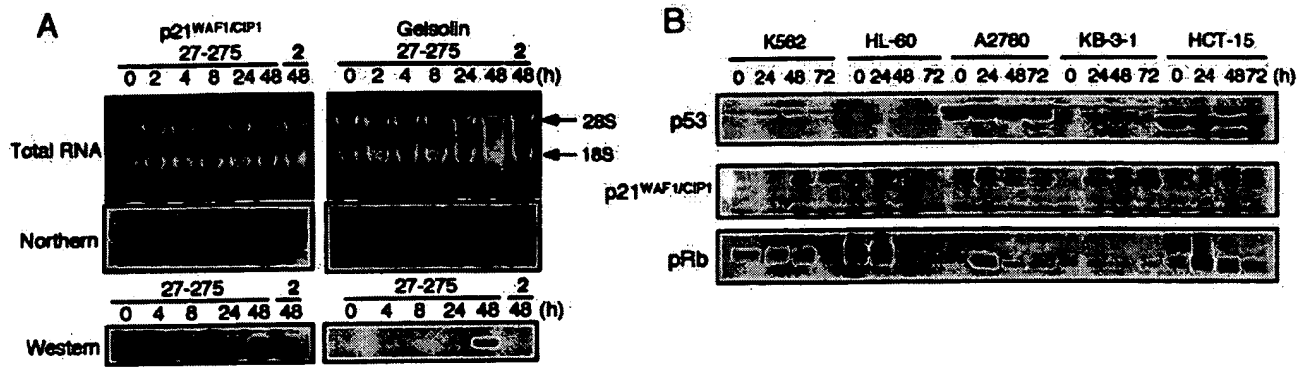

FIG. 2. Effect of MS-27-275 of $p21^{WAF1/CIP1}$ and gelsolin. (*A*) Accumulation time courses of mRNA and protein. Total RNA (10 μg) and lysate (45 μg of total protein) extracted from K562 cells exposed to 0.3 μM MS-27-275 or compound 2 for the indicated periods were used for Northern and Western blotting, respectively. (*B*) Western blot analysis of p53, $p21^{WAF1/CIP1}$, and pRb. Lysates (45 μg of total protein) of the cells exposed to 0.3 μM MS-27-275 for the indicated periods were examined by Western blot analysis by using antibodies specific to p53, $p21^{WAF1/CIP1}$, and pRb. The details are described in *Materials and Methods*.

Table 1. *In vitro* sensitivity to MS-27-275 of human tumor cell lines

Cell line	IC_{50}, μM			p53 gene status*
	MS-27-275	Compound 2	5-FU	
A2780	0.0415	0.621	13.7	wt
Calu-3	0.195	>100	41.3	-
HL-60	0.212	42.1	7.09	mu
K562	0.589	58.0	140	mu
St-4	0.820	>100	1.63	-
HT-29	1.29	>100	153	mu
KB-3-1	1.46	>100	51.8	-
Capan-1	1.70	>100	14.2	mu
4-1St	1.92	>100	144	-
HCT-15	4.71	>100	26.3	mu

The procedure to assess IC_{50} is described in *Materials and Methods*. *Citation from refs. 42–45. mu, mutant in p53 gene; wt, wild-type in p53 gene; -, the p53 gene status has not been reported.

genetic alterations, which might be acquired during cancer development, in the loci downstream of histone acetylation.

Antitumor Effect of MS-27-275 in Tumor-Bearing Nude Mice. The *in vivo* therapeutic efficacy of MS-27-275 was examined by using the eight solid human tumor lines, which showed various sensitivities as described above (Table 1). MS-27-275 at 49 mg/kg showed marked antitumor effects against KB-3-1, 4-1St, and St-4 tumor lines, and a moderate effect against Capan-1 tumor (Fig. 5*A*). The drug at 24.5 mg/kg and 12.3 mg/kg also showed significant effects against these tumors. Because the dose of 49 mg/kg was the maximum tolerated dose in this administration schedule and apparent signs of toxicity such as weight loss and poor appearance were observed, we lowered the maximum dose of the drug to 24.5 mg/kg, at which no gross weight loss was observed, for the other tumors. The dose of 24.5 mg/kg was also markedly effective against A2780 and HT-29 and moderately effective against Calu-3 (Fig. 5*A*). Among the eight tumor lines examined, only HCT-15, relatively insensitive to MS-27-275 *in vitro* (Table 1), did not respond to MS-27-275 (Fig. 5*A*). Seven of the eight tumor lines examined responded well to MS-27-275, although only one line, St-4, responded well to 5-FU at the maximum tolerated dose (30 mg/kg). Compound 2 at 98 mg/kg, 2-fold higher than the maximum tolerated dose of MS-27-275, did not show any *in vivo* therapeutic activities against two tumor lines, KB-3-1 and HT-29, that were sensitive to MS-27-275 (Fig. 5*A*). In addition, oral administration of MS-27-275 apparently increased the level of histone acetylation in HT-29 tumor xenografts 4–24 h after the administration whereas compound 2 did not show the effect (Fig. 5*B*). These result suggests that the *in vivo* therapeutic efficacies of MS-27-275 observed in this study were derived from the HDA-inhibitory action of the compound but not from unidentified cytotoxic effects.

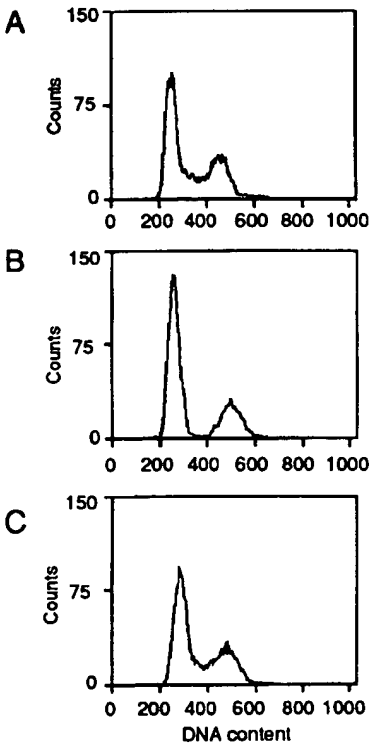

FIG. 3. Effect of MS-27-275 on cell cycle distribution in A2780 cells. Unsynchronized A2780 cells were grown in the absence (*A*) or presence of 1 μM MS-27-275 (*B*) or compound 2 (*C*) for 24 h, and the cells were harvested and examined by flow cytometry as described in *Materials and Methods*.

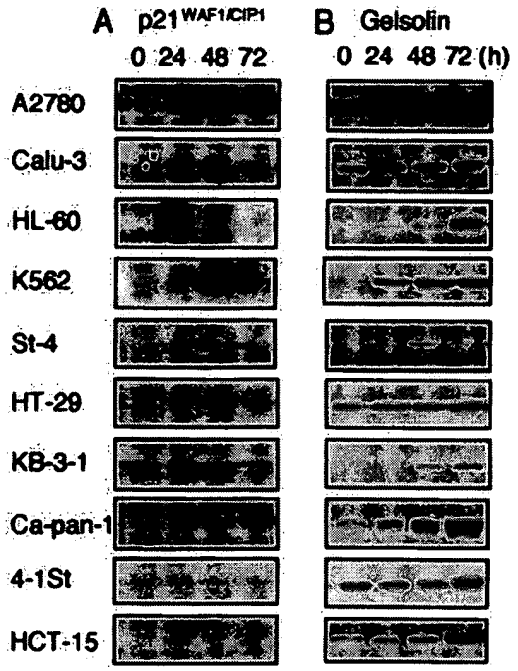

FIG. 4. Comparison of the accumulation of $p21^{WAF1/CIP1}$ (*A*) and gelsolin (*B*) in various human tumor lines exposed to MS-27-275. Lysates (45 μg of total protein) from the cells exposed to 0.3 μM MS-27-275 for 0, 24, 48, and 72 h were analyzed by Western blotting by using specific antibodies to $p21^{WAF1/CIP1}$ and gelsolin as described in *Materials and Methods*.

DISCUSSION

In the present study, we demonstrated that a synthetic compound, MS-27-275, that shares no structural similarity to other HDA inhibitors such as NaBu (11), trichostatin A (26), and trapoxin (27) revealed HDA-inhibition and antitumor efficacy. Because compound 2, a structural analogue of MS-27-275 possessing a 3′-aminophenyl group instead of a 2′-aminophenyl group, showed no inhibition of HDA, it is suggested that binding of the 2′-aminophenyl group to an unidentified but specific site on HDA molecule is important for the function of MS-27-275 as an inhibitor. Furthermore, together with the structural feature, the difference in effective concentration between MS-27-275 and the other compounds suggests the differences in the binding site on HDA molecule and the mode of action between them.

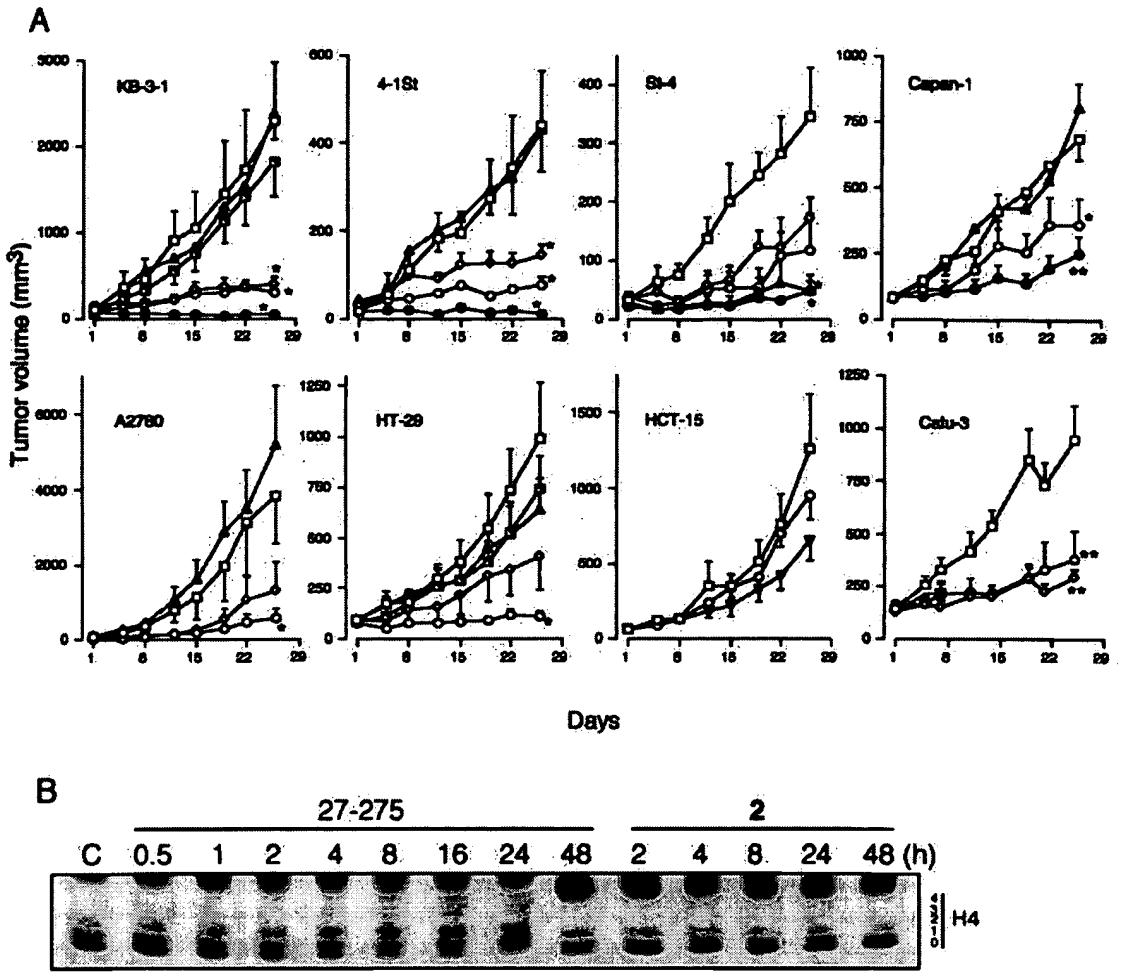

FIG. 5. *In vivo* effects of MS-27-275 against human tumor xenografts. (*A*) Antitumor efficacies against human tumor xenografts. The effects of MS-27-275 at doses of 12.3 (◇), 24.5 (○) and 49 mg/kg (●), Compound 2 at 98 mg/kg (▧), and 5-FU at 30 (▲) and 40 mg/kg (▼) were examined as described in *Materials and Methods*. □ represents the control group. Vertical bars indicate standard errors. *, $P < 0.05$; **, $P < 0.01$, respectively, by Dunnett's test or Steel's test. The discontinued line in the Calu-3 experiment was caused by death of mice in the test group. (*B*) Increase of histone acetylation in HT-29 tumor xenografts. MS-27-275 (49 mg/kg) and compound 2 (49 mg/kg) were administered orally to the nude mice with HT-29 tumors (tumor size average, 179 mm^3). At the indicated period after the administration, tumor samples were removed, and histones were extracted and analyzed as described in *Materials and Methods*. A representative of three similar observations is shown.

As shown in studies of the biological activities of the other HDA inhibitors reported recently (33–36), MS-27-275 inhibited cell proliferation and induced $p21^{WAF1/CIP1}$ and gelsolin through transcriptional activation. In all of the cell lines examined, the level of $p21^{WAF1/CIP1}$ was increased in the presence of MS-27-275, irrespective of the amount of p53 and the status of p53 gene, and the accumulation of $p21^{WAF1/CIP1}$ tended to be greater and faster in the cell lines sensitive to MS-27-275 whereas the accumulation of gelsolin seemed to have no correlation to the *in vitro* sensitivity of the cells. Because it has been reported that overexpression of gelsolin in transformed cells decreased tumorigenicity when the cells were inoculated into mice (49), gelsolin may function as a tumor suppressor in the body but not in cell culture. From these observations, it is possible that the induction of $p21^{WAF1/CIP1}$ through histone acetylation plays one of the crucial roles in the action of MS-27-275.

MS-27-275 strongly inhibited the growth in seven of eight human tumor xenografts implanted into nude mice whereas 5-FU, a commonly used agent against cancers, had a marked effect on only one tumor line. It has been known that many human tumors harbor defects in p53 genes (51, 52) and that p53 monitors the integrity of the genome and halts cell proliferation through induction of $p21^{WAF1/CIP1}$ in response to DNA damage induced by antitumor agent. Therefore, activation of this signaling pathway has been considered to be important for the efficacy of antitumor agents, and direct transactivation of the $p21^{WAF1/CIP1}$ gene bypassing p53 can serve a novel strategy for treating cancers that are insensitive to classical antitumor agents. Recently, another tumor suppressor gene, *BRCA1*, which is associated with hereditary breast and ovary cancers, was reported to activate the $p21^{WAF1/CIP1}$ gene in a p53-independent manner (53). Therefore, the induction of $p21^{WAF1/CIP1}$ by HDA inhibition may be useful for not only cancers with defects in p53 gene but also for those harboring defects in other genes controlling the expression of $p21^{WAF1/CIP1}$.

Compound 2, a structural analogue of MS-27-275 with no HDA inhibitory activity, exhibited no induction of these two proteins nor antiproliferative efficacy both *in vitro* and *in vivo*. We detected an increase in acetylation of nuclear histones in tumors implanted into mice after oral administration of MS-27-275. However, no increase in histone acetylation was observed after administration of compound 2 whereas both drugs were incorporated equally well into mouse blood by oral administration (data not shown). These results demonstrate that MS-27-275 exerts its antitumor effect through acetylation of nuclear histones. Therefore, we conclude that this com-

pound can provide a unique way to treat cancers refractory to classical antitumor agents.

1. Inoue, A. & Fujimoto, D. (1970) *Biochim. Biophys. Acta* **220,** 307–316.
2. Vidali, G., Boffa, L. C. & Allfrey, V. G. (1972) *J. Biol. Chem.* **247,** 7365–7373.
3. Kikuchi, H. & Fujimoto, D. (1973) *FEBS Lett.* **29,** 280–282.
4. Csordas, A. (1990) *Biochem. J.* **265,** 23–38.
5. Allegra, P., Sterner, R., Clayton, D. F. & Allfrey, V. G. (1987) *J. Mol. Biol.* **196,** 379–388.
6. Hebbes, T. R., Thorne, A. W. & Crane-Robinson, C. (1988) *EMBO J.* **7,** 1395–1403.
7. Tazi, J. & Bard, A. (1990) *Cell* **60,** 909–920.
8. Roth, S. Y. & Allis, C. D. (1996) *Cell* **87,** 5–8.
9. Wade, P. A., Pruss, D. & Wolfe, A. P. (1997) *Trends Biochem. Sci.* **22,** 128–132.
10. Pazin, M. J. & Kadonaga, J. T. (1997) *Cell* **89,** 325–328.
11. Riggs, M. G., Whittaker, R. G., Neuman, J. R. & Ingram, V. M. (1977) *Nature (London)* **268,** 462–464.
12. Cioe, L., McNab, A., Hubbell, H. R., Meo, P., Curtis, P. & Rovera, G. (1981) *Cancer Res.* **41,** 237–243.
13. Hoessly, M. C., Rossi, R. M. & Fischkoff, S. A. (1989) *Cancer Res.* **49,** 3594–3597.
14. Kim, Y. S., Tsao, D., Siddiqui, B., Whitehead, J. S., Arnstein, P., Bennet, J. & Hicks, J. (1980) *Cancer* **45,** 1185–1192.
15. Tsao, D., Morita, A., Bella, A., Luu, P. & Kim, Y. S. (1982) *Cancer Res.* **42,** 1052–1058.
16. Ryan, M. P., Borenfreund, E. & Higgins, P. J. (1987) *J. Natl. Cancer Inst.* **79,** 555–567.
17. Graham, K. A. & Buick, R. N. (1988) *J. Cell. Physiol.* **136,** 63–71.
18. Guilbaud, N. F., Gas, N., Dupont, M. A. & Valette, A. (1990) *J. Cell. Physiol.* **145,** 162–172.
19. Ryan, M. P. & Higgins, P. J. (1988) *J. Cell. Physiol.* **137,** 25–34.
20. Huang, M. E., Ye, Y. C., Chen, S. R., Chai, J. R., Lu, J. X., Zhoa, L., Gu, L. J. & Wang, Z. Y. (1988) *Blood* **72,** 567–572.
21. Novogradsky, A., Dvir, A., Ravid, A., Shkolnik, T., Stenzel, K. H., Rubin, A. L. & Zaizov, R. (1983) *Cancer* **51,** 9–14.
22. Miller, A. A., Kurschel, E., Osieka, R. & Schmidt, C. (1987) *Eur. J. Cancer Clin. Oncol.* **23,** 1283–1287.
23. Pouillart, P., Cerutti, I., Ronco, G., Villa, P. & Chany, C. (1991) *Int. J. Cancer* **49,** 89–95.
24. Planchon, P., Pouillart, P., Ronco, G., Villa, P. & Pieri, F. (1993) *J. Pharm. Sci.* **82,** 1046–1048.
25. Rephaeli, A., Rabizadeh, E., Aviram, A., Shaklai, M., Ruse, M. & Nudelman, A. (1991) *Int. J. Cancer* **49,** 66–72.
26. Yoshida, M., Kijima, M., Akita, M. & Beppu, T. (1990) *J. Biol. Chem.* **265,** 17174–17179.
27. Kijima, M., Yoshida, M., Sugita, K., Horinouchi, S. & Beppu, T. (1993) *J. Biol. Chem.* **268,** 22429–22435.
28. Kwon, H. J., Owa, T., Hassig, C. A., Shimada, J. & Schreiber, S. L. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3356–3361.
29. Sato, W., Fukazawa, N., Nakanishi, O., Baba, M., Suzuki, T., Yano, O., Naito, M. & Tsuruo, T. (1995) *Cancer Chemother. Pharmacol.* **35,** 271–277.
30. Baba, M., Nakanishi, O., Saito, A., Miyama, Y., Yano, O., Shimada, S., Fukazawa, N., Naito, M. & Tsuruo, T. (1995) *Cancer Chemother. Pharmacol.* **36,** 361–367.
31. Nakanishi, O., Baba, M., Saito, A., Yamashita, T., Sato, W., Abe, H., Fukazawa, N., Suzuki, T., Sato, S., Naito, M., *et al.* (1997) *Oncol. Res.* **9,** 61–69.
32. Suzuki, T., Ando, T., Tsuchiya, K., Nakanishi, O., Saito, A., Yamashita, T., Shiraishi, Y. & Tanaka, E. (1997) Eur. Patent 847992; (1998) *Chem. Abstr.* **29,** 95402.
33. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsuzawa, Y., Tokino, T., *et al.* (1997) *J. Biol. Chem.* **272,** 22199–22206.
34. Siavoshian, S., Blottiere, H. M., Cherbut, C. & Galmiche, J. P. (1997) *Biochem. Biophys. Res. Commun.* **232,** 169–172.
35. Higgins, P. J. & Ryan, M. P. (1991) *Biochem. J.* **279,** 883–890.
36. Hoshikawa, Y., Kwon, H. J., Yoshida, M., Horinouchi, S. & Beppu, T. (1994) *Exp. Cell Res.* **214,** 189–197.
37. Harper, J. W., Hannon, G. J., Zhang, H., Casso, D. & Elledge, S. J. (1993) *Cell* **75,** 805–816.
38. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. & Beach, D. (1993) *Nature (London)* **366,** 701–704.
39. Gu, Y., Turck, C. W. & Morgan, D. O. (1993) *Nature (London)* **366,** 707–710.
40. Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J. & Reed, S. I. (1994) *Cell* **76,** 1013–1023.
41. El-Diery, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E. & Vogelstein, B. (1993) *Cell* **75,** 817–825.
42. Wolf, D. & Rotter, V. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 790–794.
43. O'Connor, P. M., Jackman, J., Bae, I., Myers, T. G., Fan, S., Mutoh, M., Scudiero, D. A., Monks, A., Sausville, E. A., Weinstein, J. N., *et al.* (1997) *Cancer Res.* **57,** 4285–4300.
44. Brown, R., Clugston, C., Burns, P., Edlin, A., Vasey, P., Vojtesek, B. & Kaye, S. B. (1993) *Int. J. Cancer* **55,** 678–684.
45. Ruggeri, B., Zhang, S., Caamano, J., DiRado, M., Flynn, S. D. & Klein Szanto, A. J. (1992) *Oncogene* **7,** 1503–1511.
46. DeCaprio, J., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M. & Livingston, D. M. (1989) *Cell* **58,** 1085–1095.
47. Buchkovich, K., Duffy, L. A. & Harlow, E. (1989) *Cell* **58,** 1097–1105.
48. Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y. J. & Lee, W. H. (1989) *Cell* **58,** 1193–1198.
49. Müllauer, L., Fujita, H., Ishizaki, A. & Kuzumaki, N. (1993) *Oncogene* **8,** 2531–2536.
50. Tanaka, M., Müllauer, L., Ogiso, Y., Fujita, H., Moriya, S., Furuuchi, K., Harabayashi, T., Shinohara, N., Koyanagi, T. & Kuzumaki, N. (1995) *Cancer Res.* **55,** 3228–3232.
51. Vogelstein, B., Fearson, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M. & Bos, J. L. (1988) *N. Engl. J. Med.* **319,** 525–532.
52. Vogelstein, B. & Kinzler, K. W. (1998) *Cell* **70,** 523–526.
53. Somasdaram, K., Zhang, H., Zeng, Y.-X., Houvras, Y., Peng, Y., Zhang, H., Wu, G. S., Licht, J. D., Weber, B. L. (1997) *Nature (London)* **389,** 187–190.

Exhibit 3

REVIEW

Histone Deacetylase Inhibitors: Inducers of Differentiation or Apoptosis of Transformed Cells

Paul A. Marks, Victoria M. Richon, Richard A. Rifkind

Histone deacetylase (HDAC) inhibitors have been shown to be potent inducers of growth arrest, differentiation, and/or apoptotic cell death of transformed cells *in vitro* and *in vivo*. One class of HDAC inhibitors, hydroxamic acid-based hybrid polar compounds (HPCs), induce differentiation at micromolar or lower concentrations. Studies (x-ray crystallographic) showed that the catalytic site of HDAC has a tubular structure with a zinc atom at its base and that these HDAC inhibitors, such as suberoylanilide hydroxamic acid and trichostatin A, fit into this structure with the hydroxamic moiety of the inhibitor binding to the zinc. HDAC inhibitors cause acetylated histones to accumulate in both tumor and normal tissues, and this accumulation can be used as a marker of the biologic activity of the HDAC inhibitors. Hydroxamic acid-based HPCs act selectively to inhibit tumor cell growth at levels that have little or no toxicity for normal cells. These compounds also act selectively on gene expression, altering the expression of only about 2% of the genes expressed in cultured tumor cells. In general, chromatin fractions enriched in actively transcribed genes are also enriched in highly acetylated core histones, whereas silent genes are associated with nucleosomes with a low level of acetylation. However, HDACs can also acetylate proteins other than histones in nucleosomes. The role that these other targets play in the induction of cell growth arrest, differentiation, and/or apoptotic cell death has not been determined. Our working hypothesis is that inhibition of HDAC activity leads to the modulation of expression of a specific set of genes that, in turn, result in growth arrest, differentiation, and/or apoptotic cell death. The hydroxamic acid-based HPCs are potentially effective agents for cancer therapy and, possibly, cancer chemoprevention. [J Natl Cancer Inst 2000;92:1210–6]

Neoplastic transformation is characterized by inappropriate cell proliferation and/or altered patterns of cell death. However, neoplastic transformation does not necessarily destroy the potential for expression of differentiated characteristics, including cessation of proliferation under appropriate environmental conditions *(1)*. For example, cells infected with temperature-sensitive transforming viruses *(2)* can display either normal or transformed properties, depending on the activity of a temperature-sensitive viral protein. Some malignant cells (e.g., from teratocarcinomas, neuroblastomas, or leukemias) can differentiate along apparently normal pathways when placed in a normal embryonic environment *(3–7)*. In addition, various chemical agents [hybrid polar compounds (HPCs) *(8–10)*, retinoids *(11–15)*, vitamin D_3 *(16)*, and several other agents *(17–19)*] can induce certain transformed cells *in vitro* to express differentiated characteristics and stop proliferating.

Histones are part of the core proteins of nucleosomes. Acetylation and deacetylation of these proteins play a role in the regulation of gene expression *(20)*. There are two classes of enzymes involved in determining the state of acetylation of histones, histone acetyl transferases (HATs) and histone deacetylases (HDACs). There are several reports *(21–24)* that altered HAT or HDAC activity is associated with cancers.

During the last decade, a number of HDAC inhibitors have been identified that induce cultured tumor cells to undergo growth arrest, differentiation, and/or apoptotic cell death *(25–35)*. These agents also inhibit the growth of cancer cells in animal models *(32,35–40)*, and several agents, in particular, hydroxamic acid-based HDAC inhibitors, inhibit tumor growth in animals at doses that are apparently nontoxic and appear to be selective.

This review focuses on studies of HDAC inhibitors, especially on the hydroxamic acid-based HPCs. These compounds represent a class of agents that are potentially effective cancer therapies. (Studies were identified for this review by searching the MEDLINE® database for appropriate papers published in the last 10 years and by a review of bibliographies from articles identified through that search. In addition, we include some of our unpublished data.)

Histone Acetylation and Deacetylation and Gene Expression

Structure of Nucleosomes

Analyses (x-ray and electron crystallographic) show that nucleosomes contain an average of 150 base pairs of DNA wrapped around the nucleosomal core of histones in 1.75 turns of left-handed superhelical DNA *(41–43)*. Five classes of histones have been identified in chromatin: histones H1, H2A, H2B, H3, and H4. Each nucleosome contains two H2As, two H2Bs, two H3s, and two H4s in the core (Fig. 1). Histone H1 occurs in chromatin in about half the amount of the other types of histones and appears to lie on the outer portion of the nucleosome.

Role of Histone Acetylases and Deacetylases

Histones of the nucleosomal core can be acetylated and deacetylated, and the amount of acetylation is controlled by the opposing activities of two types of enzymes, HATs and HDACs. Substrates for these enzymes include ε-amino groups of lysine

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See "Notes" following "References."

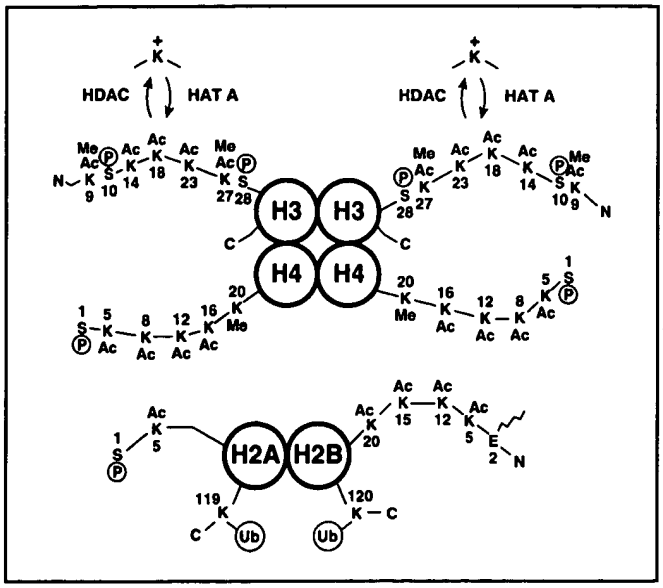


Fig. 1. Histones in nucleosomes. Lysines (K) in the amino (N)-terminal tails of histones H3, H4, H2A, and H2B are potential acetylation/deacetylation sites for histone acetyltransferase (HAT) and histone deacetylase (HDAC). K^+ = positively charged lysine, Ub = ubiquitin, P = phosphate, Ac = acetyl, S = serine, E = glutamic acid, and Me = methyl. [Adapted with permission from Davie *(44).*]

residues located in the amino-terminal tails of the histones. When HDAC removes the acetyl group from histone lysine, it restores a positive charge to the lysine residue condensing the structure of nucleosomes *(44).*

HATs and HDACs

There are at least four groups of proteins with intrinsic HAT activity *(45–50).* The first group contains the GCN5 and P/CAF proteins, which are related to yeast HAT GCN5. The second group contains the closely related cyclic adenosine monophosphate response element-binding protein (CBP) and p300, which act as coactivators for a number of transcription factor complexes. The third group contains the TAF250 protein, part of the basic transcription complex TFIID that binds the TATA box. The fourth group contains the SRC-1 and ACTR proteins that are coactivators for ligand-activated nuclear receptors. In addition, there are probably several other proteins with HAT activity, such as BRCA2, that are part of transcription complexes. HATs play a role in activation of gene expression and may also be involved in gene repression, as suggested by the observation in *Drosophila* that acetylation of the transcription factor T-cell factor by CBP represses transcription *(51).*

Eight HDACs have been described in mammalian cells *(45,52–59).* The yeast RPD3 homologues are HDAC1, HDAC2, HDAC3, and HDAC8; the yeast HDA1 homologues are HDAC4 (also known as HDAC-A), HDAC5 (also known as mHDA1), HDAC6 (also known as mHDAC2), and HDAC7.

Regulation of Transcription

Chromatin fractions enriched in actively transcribed genes are also enriched in highly acetylated core histones *(20,42,45),* whereas silent genes are associated with nucleosomes with a low level of acetylation. Allfrey *(60)* first suggested that histone acetylation was involved in the regulation of transcription. During the past decade, considerable evidence has accumulated to establish the role of acetylation and deacetylation of histones in the regulation of transcription *(20,41–43,45).* The following model describes a role for histone acetylation in regulating gene transcription. Nucleosomes containing highly charged hypoacetylated histones bind tightly to the phosphate backbone of DNA, inhibiting transcription, presumably, because transcription factors, regulatory complexes, and RNA polymerase do not have access to the DNA. Acetylation neutralizes the charge of the histones and generates a more open DNA conformation. Transcription factors and the transcription apparatus then have access to the DNA, and expression of the corresponding genes is promoted (Fig. 2).

In addition to HDACs and HATs, other factors are involved in the regulation of chromatin structure, including methyl-CpG-binding protein *(61–63)* and adenosine triphosphate (ATP)-dependent chromatin-remodeling complexes *(64).* These chromatin-modifying complexes interact with HAT and HDAC complexes to regulate transcriptional activity of genes [for a recent review of chromatin methylation, *see (63);* for reviews of the ATP-dependent chromating remodeling complexes, *see (64,65).*]

HDACs are bound to large protein complexes that regulate gene transcription. Mammalian HDAC1 and HDAC2 are associated with the Sin3 complex that includes NCo-R, SMRT, and several other, as yet, unidentified proteins and appear to repress gene expression by deacetylating core histones. In addition to deacetylation of histones, HDACs may also regulate gene expression by deacetylating transcription factors, such as p53, GATA-1, TFIIE, and TFIIF *(66–68).* HDACs may also participate in cell cycle regulation. The transcription repression mediated by RB binding to the transcription factor E2F involves recruitment of HDAC1 or HDAC2 by RB *(69,70).*

Disruption of HAT and/or HDAC Activity and Development of Cancer

Mutations in the CBP gene, which encodes an HAT, are associated with leukemogenesis and the developmental disorder

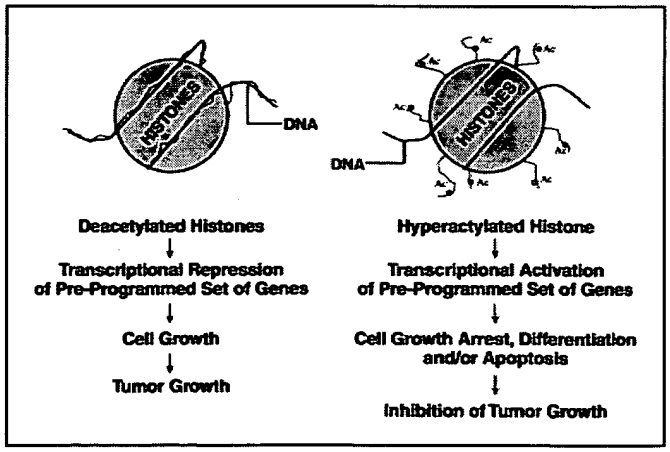


Fig. 2. Proposed mechanism of action of histone deacetylase (HDAC) inhibitors that induce tumor growth arrest, differentiation, and/or apoptotic cell death. With inhibition of HDAC, histones are acetylated (Ac), and the DNA that is tightly wrapped around a deacetylated histone core relaxes. We propose that the accumulation of acetylated histones in nucleosomes leads to expression of specific genes, which, in turn, lead to cell growth arrest, differentiation, and/or apoptotic cell death and, as a consequence, inhibition of tumor growth.

Rubinstein–Taybi syndrome *(71)*. Patients with Rubinstein–Taybi syndrome have a propensity to develop cancer. Microdeletions, translocations, inversions, and various point mutations in the CBP gene have been identified in patients with Rubinstein–Taybi syndrome as well as in patients with some types of colorectal or gastric carcinomas *(21)*. Gene fusions with CBP are associated with several leukemias. In therapy-related acute myeloid and lymphoid leukemias and in myelodysplasia, the CBP gene has been found fused to the MLL gene, and the CBP gene has been found fused in acute myeloid leukemia to the MOZ gene *(72,73)*.

Several leukemogenic transcription factors repress expression of specific genes because of aberrant recruitment of HDACs. This repression of gene expression appears to be an important step in the leukemogenic action of these transcription factors. For example, aberrant recruitment of HDAC activity has been reported in cell lines derived from patients with acute promyelocytic leukemia (APL) *(22–24)*. The oncoprotein encoded by the translocation-generated fusion gene in APL (promyelocytic leukemia [PML]–retinoic acid receptor-α) represses transcription by recruitment of HDAC1. Furthermore, resistance to the differentiating actions of all-*trans*-retinoic acid in a patient with APL was overcome by cotreatment with an inhibitor of HDAC *(74)*. [In a further study *(75)*, four other patients with APL failed to respond.] HDAC-dependent aberrant transcriptional repression is implicated as the main oncogenic mechanism in specific types of myeloid leukemia and lymphoma. For example, in non-Hodgkin's lymphoma, the transcriptional repressor BCL6 is inappropriately overexpressed within the lymphoid compartment, resulting in aberrant transcriptional repression and lymphoid oncogenic transformation *(76)*. Another example is acute myelogenous leukemia of the M2 subtype associated with the t(8;21) chromosomal translocation involving the AML1 and ETO genes *(77)*. The AML1–ETO fusion protein, unlike the AML-1 protein (a transcriptional activator), is a potent dominant transcriptional repressor. In both of these cases, transcriptional repression appears to be mediated by recruitment of HDAC to the transcriptional repressor complex.

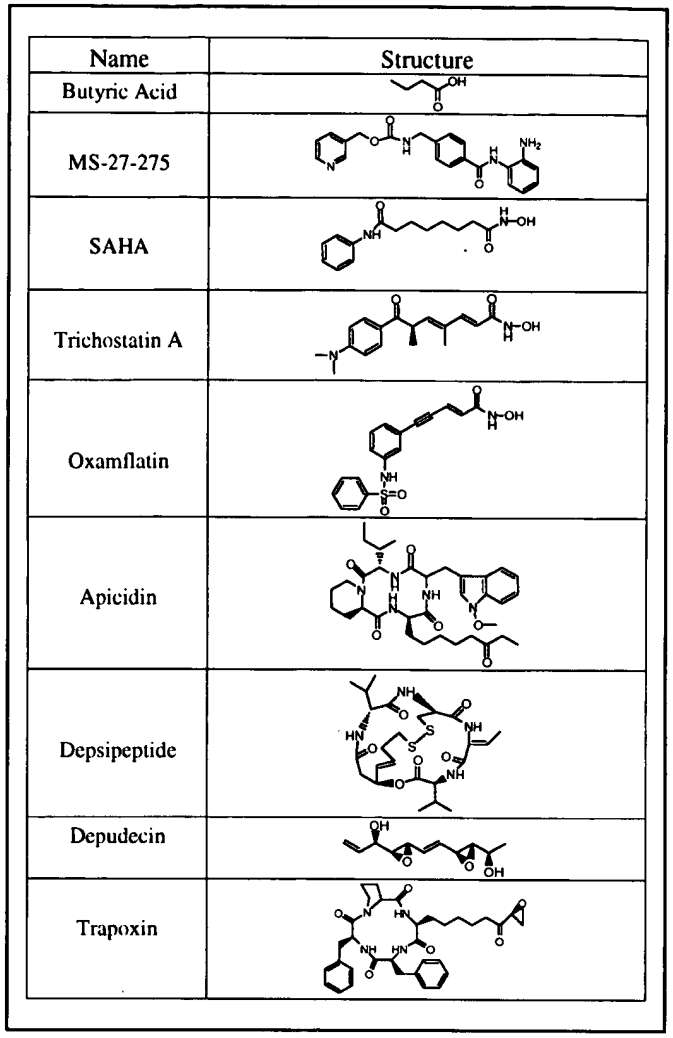
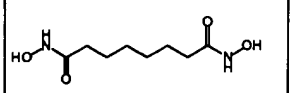
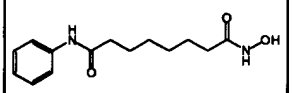
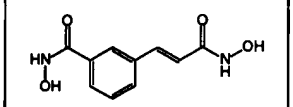
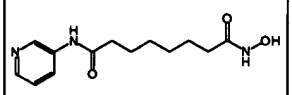


Fig. 3. Histone deacetylase inhibitors (*see* text for references to these inhibitors). SAHA = suberoylanilide hydroxamic acid.

HDAC Inhibitors

Compounds that inhibit HDAC activity are shown in Fig. 3. Several structural classes of HDAC inhibitors have been identified including the following: 1) short-chain fatty acids [e.g., butyrates *(28,31)*]; 2) hydroxamic acids [e.g., trichostatin A (TSA) *(25,26)*, suberoylanilide hydroxamic acid (SAHA) *(34)*, and oxamflatin *(35)*]; 3) cyclic tetrapeptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl (AOE) moiety [e.g., trapoxin A) *(27)*]; 4) cyclic peptides not containing the AOE moiety [e.g., FR901228 and apicidin *(33,78)*]; and 5) benzamides [e.g., MS-27-275 *(32)*]. HDAC inhibitors invariably inhibit proliferation of transformed cells in culture, and a subset has been shown to inhibit tumor growth in animal models *(26,32,35–40)*. The butyrates represent the only class that is approved currently for use in the clinic. The butyrates are not ideal agents because of the high concentrations required (millimolar) to achieve inhibition of HDAC activity and multiple effects on other enzyme systems *(28,31)*. TSA, originally developed as an antifungal agent *(25,26,29)*, is a potent inhibitor of HDAC that is active at nanomolar concentrations. The finding that TSA-resistant cell lines have an altered HDAC is evidence that this enzyme is an important target for TSA. Oxamflatin *(35)*, a hydroxamic acid-based compound, and the benzamide MS-27-275 *(32)* inhibit HDAC activity at micromolar concentrations. Apicidin is a fungal metabolite that exhibits potent, broad-spectrum antiprotozoal activity and inhibits HDAC activity at nanomolar concentrations *(78)*. Depsipeptide (FR901228), isolated from *Chromobacterium violaceum (33)*, inhibits HDAC activity at micromolar concentrations. Trapoxin *(27)* and depudecin *(30)* irreversibly bind to HDAC and inhibit its activity at nanomolar and micromolar concentrations, respectively.

In our laboratory, a series of hydroxamic acid-based HPCs have been synthesized that inhibit HDACs at micromolar concentrations or lower *in vitro* and *in vivo (34,36,38,79)* (Fig. 4), and extensive structure–activity studies have been done with these compounds *(34,79)*. The essential characteristics of hydroxamic acid-based HPCs are a polar site, the hydroxamic group, a six-carbon hydrophobic methylene spacer, a second polar site, and a terminal hydrophobic group (Fig. 4). Substitution of the hydroxamic acid with a carboxylic acid or amide oxime group results in inactive compounds. Modification of the hydroxamic acid, such as introduction of a methyl group on an adjacent carbon or *N*-methylation, results in inactive compounds. The benzene ring in the hydrophobic moiety can be

Name	Structure	Opt. Conc.	% Diff.
SBHA		30 μM	90%
SAHA		2.5 μM	68%
CBHA		4.0 μM	73%
Pyroxamide		4.0 μM	51%

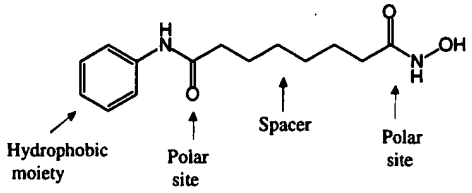


Fig. 4. Hydroxamic acid-based hybrid polar compounds. The optimal concentration to induce murine erythroleukemia cells to differentiate (% Diff) was determined from the percent of differentiated cells [detected as benzidine-stained cells (benzidine binds to the iron-containing heme of hemoglobin); for details of methods, *see (34)*]. SBHA = suberic bishydroxamic acid; SAHA = suberoylanilide hydroxamic acid; CBHA = *m*-carboxy-cinnamic acid bishydroxamic acid.

modified in the meta and para positions without loss of activity; however, in general, larger substituents are associated with loss of activity. The optimal methylene spacer is six methylenes, five- and seven-carbons spacers being less active.

The structure of the catalytic core of HDACs has been determined by x-ray crystallography *(80)*. HDACs share an approximately 390-amino acid region of homology, referred to as the deacetylase core. Residues that form the active site are conserved across all HDACs. The deacetylase core identifies a gene superfamily that includes an HDAC homologue in the hyperthermophilic bacterium *Aquifex aeolicus* (termed "HDLP"), which was used for x-ray crystallography studies. There is a 35.2% base-pair identity between sequences of the catalytic core of the HDLP and of the mammalian HDAC1. HDLP deacetylates histones *in vitro*, its activity is inhibited by TSA and SAHA, but its specific activity is equal to about 7.5% of that of partially purified HDAC1. From x-ray crystallographic analyses of HDLP, an HDLP–TSA complex, and an HDLP–SAHA complex, the active catalytic site in the HDLP was shown to be formed by a tubular pocket, a zinc-binding site, and two asparagine–histidine charge-relay systems (Fig. 5). The hydroxamic acid moieties of TSA and SAHA bind to the zinc in the tubular pocket and the carbon-ring group projects out of the pocket on the surface of the protein.

Activity of HDAC Inhibitors *In Vitro*

The hydroxamic acid-based HPCs (e.g., *m*-carboxy-cinnamic acid bishydroxamic acid [CBHA], suberic bishydroxamic acid [SBHA], SAHA, and pyroxamide) (Fig. 4) inhibit partially purified HDAC1 and HDAC3 at concentrations of 0.01–1.0 μ*M* *(34)*. Furthermore, the optimal concentrations of various HPCs that induce murine erythroleukemia (MEL) cell differentiation as assayed by the proportion of cells that become benzidine positive (a stain for heme of hemoglobin) are correlated directly with the concentration required to inhibit the activity of partially purified HDAC1 or HDAC3 over a wide concentration range.

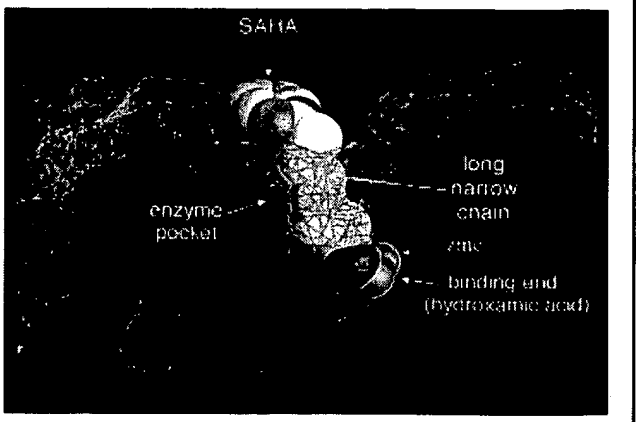


Fig. 5. SAHA (suberoylanilide hydroxamic acid) binds to the pocket of the catalytic site of a histone deacetylase-like protein, schematically represented by the netting. SAHA makes contact with residues at the rim, walls, and bottom of the pocket (enzyme pocket). The hydroxamic acid moiety of SAHA binds to the zinc at the bottom of the pocket *(80)*. (The figure is courtesy of Michael S. Finnin and Nikola P. Pavletich.)

With the use of MEL cells and T24 human bladder carcinoma cells in culture, the effects of SAHA and related hydroxamic acid-based HPCs on the acetylation of histones have been examined *(34)*. SAHA, pyroxamide, SBHA, and CBHA (Fig. 4) cause accumulation of acetylated histones. Acetylated histone type-specific antibodies were used to show that, when cells were cultured with hydroxamic acid-based HPCs, the level of acetylation in histones H2A, H2B, H3, and H4 increased (Fig. 6). Increased histone acetylation could be detected as early as 1 hour after MEL or T24 cells were cultured with SAHA or other hydroxamic acid-based HPCs. The level of acetylated histones reached a maximum 6–12 hours after the addition of HPCs and remained elevated as long as the HPC was present *(34)*.

HDAC inhibitors can induce growth arrest, differentiation, and/or apoptotic cell death in a wide variety of cultured transformed cells, including neuroblastoma, melanoma, and leukemia cells, as well as cells from breast, prostate, lung, ovary, and colon cancers *(25–30,36,40,78,81)*. For example, SAHA induces terminal cell differentiation in several cell lines, including MEL, T24 human bladder carcinoma, and MCF-7 human breast adenocarcinoma. Differentiation was evaluated by parameters that included morphology, arrest in G_1 phase of the cell cycle, and developmental markers, such as hemoglobin in MEL cells, milk proteins in MCF-7 cells, and gelsolin in T24 cells. SAHA induces apoptotic death of human multiple myeloma cells (ARP-1), human prostate cell lines (LNCaP), and myelomonocytic leukemia cells (U937). CBHA induced apoptotic cell death of several human neuroblastoma cell lines, LAI-55n, KCN-69n, and SK-N-ER. Apoptosis was assayed by DNA fragmentation analysis and the deletion of a sub-G_1 (<2N ploidy) population by flow cytometry.

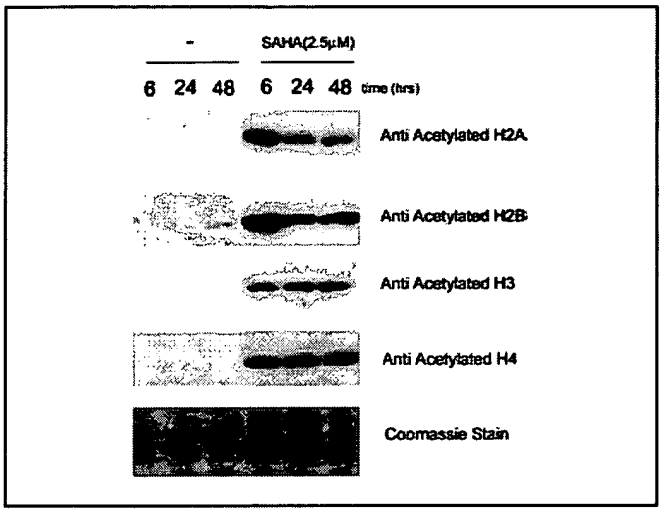


Fig. 6. Effect of SAHA (suberoylanilide hydroxamic acid) on histone acetylation in MEL cells. Cells were cultured without (–) or with 2.5 μ*M* SAHA for the times indicated. The acetylation of the histones was analyzed by use of antibodies specific for acetylated H2A, H2B, H3, and H4. The Coomassie-stained gel, **at the bottom,** indicates that the amount of protein loaded in each lane was similar [for details of methods, *see (36)*].

Van Lint et al. *(82)* have shown that the action of HDAC inhibitors on gene expression is selective. In cells cultured with TSA, the expression of only about 2% of expressed genes is changed (increased or decreased) twofold or more compared with untreated control cells. Our laboratory has obtained comparable results with transformed cells cultured with SAHA. The basis for the gene selectivity of SAHA or TSA is not known.

One gene most consistently induced by HDAC inhibitors is the cyclin-dependent kinase inhibitor $p21^{WAF1}$, which plays an important, if not determinant, role in the arrest of cell growth. Butyrate, TSA, depsipeptide, oxamflatin, MS-27-275, and the hydroxamic acid-based HPCs *(28,31,32,34,83)* induce $p21^{WAF1}$ transcription. The relation between SAHA-mediated histone hyperacetylation and increased $p21^{WAF1}$ gene expression has been studied in T24 human bladder carcinoma cells *(84)*. Increased transcription of the $p21^{WAF1}$ gene is associated with an increased level of acetylation on histones associated with the $p21^{WAF1}$ gene.

In Vivo Studies With HDAC Inhibitors

The butyrate analogue phenylbutyrate gave mixed results when tested as an HDAC inhibitor in animals and in a patient with APL. It was ineffective to moderately effective in inhibiting growth of solid tumors or leukemias, and that activity was observed only at relatively high doses *(28)*. A 13-year-old girl with relapsed APM who no longer responded to treatment with retinoic acid alone was treated with retinoic acid plus phenylbutyrate and had a complete clinical remission that was sustained for 7 months, during five treatment courses, before relapsing and becoming resistant to this treatment *(74)*. The acetylation of histones in her mononuclear blood cells was elevated during the period of administration of the phenylbutyrate. No remissions were induced in four other patients with APL *(75)*.

Several other HDAC inhibitors, including depsipeptide *(32)*, oxamflatin *(35)*, MS-27-275 *(32)*, and the hydroxamic acid-based HPCs *(37–39)*, inhibit tumor growth in animal models (Figs. 3 and 4). TSA did not inhibit the growth of a human melanoma xenograft in nude mice, but azeloic bishydroxamate did *(40)*. Treatment with HDAC inhibitors can increase the accumulation of acetylated histone in tumor tissue and/or normal tissues (e.g., spleen, bone marrow cells, and peripheral mononuclear cells). Thus, the level of acetylated histones is a useful intermediary marker of HDAC inhibitor activity.

Hydroxamic acid-based HPCs (Fig. 4) have been tested extensively in animal studies. One study *(37)* used rats with *N*-methylnitrosourea-induced mammary carcinomas. When these rats were fed SAHA (900 parts/million, continuously, beginning 7 days before the administration of *N*-methylnitrosourea), the incidence of mammary tumors was reduced by 40%, and the mean tumor volume was reduced by 78%—without side effects. Another study *(39)* used mice in which the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induces lung tumors. When these mice were fed SAHA (900 parts/million, continuously, beginning 7 days before administration of the carcinogen to the end of the studies), the formation of lung tumors was substantially inhibited—also without toxic effects. A third rodent study *(38)* used nude mice bearing transplanted CWR22 androgen-dependent human prostate tumors. When these mice were given SAHA (25, 50, or 100 mg/kg per day) daily by intraperitoneal injection for 3 weeks, starting as soon as palpable tumors were detected, SAHA suppressed tumor growth at all three doses. With doses of 50 and 100 mg/kg per day, the mean tumor volume was reduced by 97%. Acetylation of histones H3 and H4 increased in the CWR22 tumor cells within 6 hours after SAHA was injected. Pyroxamide had similar effects on CWR22 tumor growth and the accumulation of acetylated histones (Fig. 4). When SAHA or pyroxamide was given at doses that markedly inhibited tumor growth, no toxicity, as evaluated by weight gain and histologic examination of multiple tissues at necropsy, was detected.

Conclusions

The studies summarized in this review indicate that the hydroxamic acid-based HPCs, in particular, SAHA and pyroxamide—are potent inhibitors of HDAC *in vitro* and *in vivo* and induce growth arrest, differentiation, or apoptotic cell death of transformed cells. We suggest that inhibition of HDAC activity leads to relaxation of the structure of chromatin associated with a specific set of programmed genes. The relaxed chromatin structure allows these genes to be expressed, which, in turn, arrests tumor cell growth. SAHA and pyroxamide are lead compounds among the family of hydroxamic acid-based HPCs and are currently in phase I clinical trials.

References

(1) Marks PA, Rifkind RA. Erythroleukemic differentiation. Annu Rev Biochem 1978;47:419–48.

(2) Beug H, Blundell PA, Graft T. Reversibility of differentiation and proliferative capacity in avian myelomonocytic cells transformed by tsE26 leukemia virus. Genes Dev 1987;1:277–86.

(3) Brinster RL. The effect of cells transferred into the mouse blastocyst on subsequent development. J Exp Med 1974;140:1049–56.

(4) DeCosse JJ, Gossens CL, Kuzma JF, Unsworth BR. Breast cancer: induction of differentiation by embryonic tissue. Science 1973;181:1057–8.

(5) Gootwine E, Webb CG, Sachs L. Participation of myeloid leukaemic cells injected into embryos in haematopoietic differentiation in adult mice. Nature 1982;299:63–5.

(6) Illmensee K, Mintz B. Totipotency and normal differentiation of single

teratocarcinoma cells cloned by injection into blastocysts. Proc Natl Acad Sci U S A 1976;73:549–53.

(7) Brinster RL. The effects of cells transferred into mouse blastocyst on subsequent development. J Exp Med 1974;140:1049–56.

(8) Friend C, Scher W, Holland JG, Sato T. Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro:* stimulation of erythroid differentiation by dimethyl sulfoxide. Proc Natl Acad Sci U S A 1971;68:378–82.

(9) Reuben RC, Wife RL, Breslow R, Rifkind RA, Marks PA. A new group of potent inducers of differentiation in murine erythroleukemia cells. Proc Natl Acad Sci U S A 1976;73:862–6.

(10) Marks PA, Richon VM, Kiyokawa H, Rifkind RA. Inducing differentiation of transformed cells with hybrid polar compounds: a cell cycle-dependent process. Proc Natl Acad Sci U S A 1994;91:10251–4.

(11) Lotan R. Different susceptibilities of human melanoma and breast carcinoma cell lines to retinoic acid-induced growth inhibition. Cancer Res 1979;39:1014–9.

(12) Reiss M, Pitman SW, Sartorelli AC. Modulation of the terminal differentiation of human squamous carcinoma cells *in vitro* by all-*trans*-retinoic acid. J Natl Cancer Inst 1985;74:1015–23.

(13) Strickland S, Mahdavi V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell 1978;15:393–403.

(14) Sidell N. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells *in vitro*. J Natl Cancer Inst 1982; 68:589–96.

(15) Takenaga K, Hozumi M, Sakagami Y. Effects of retinoids on induction of differentiation of cultured mouse myeloid leukemia cells. Cancer Res 1980; 40:914–9.

(16) Zhou JG, Norman AW, Lubbert M, Collins ED, Uskokovic MR, Koeffler HP. Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absolution or bone mobilization. Blood 1989;74:82–93.

(17) Michaeli J, Marks PA, Rifkind RA. Differentiating agents in cancer therapy. In: Pinedo HM, Longo D, Chabner BA, editors. Cancer chemotherapy and biological response modifiers. Annual 13. Amsterdam (The Netherlands): Elsevier; 1992. p. 286–307.

(18) Huberman E, Callabaum MF. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents. Proc Natl Acad Sci U S A 1979;76:1293–7.

(19) Pierce GB, Speers WC. Tumors as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation. Cancer Res 1988;48:1996–2004.

(20) Grunstein M. Histone acetylation and chromatin structure and transcription. Nature 1997;389:349–52.

(21) Muraoka M, Konishi M, Kikuchi-Yanoshita R, Tanaka K, Shitara N, Chang JM, et al. p300 gene alterations in colorectal and gastric carcinomas. Oncogene 1996;12:1565–9.

(22) He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M, Zelent A, et al. Distinct interactions of PML-RARalpha and PLZF-RARalpha with corepressors determine differential responses to RA in APL. Nat Genet 1998; 18:126–35.

(23) Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, et al. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature 1998;391:815–8.

(24) Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr, Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 1998;391:811–4.

(25) Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, Koizumi K. A new antifungal antibiotic, trichostatin. J Antibiot (Tokyo) 1976;29:1–6.

(26) Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem 1990;265:17174–9.

(27) Kijima M, Yoshida M, Sugita K, Horinouchi S, Beppu T. Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J Biol Chem 1993;268:22429–35.

(28) Newmark HL, Lupton JR, Young CW. Butyrate as a differentiating agent: pharmacokinetics, analogues and current status. Cancer Lett 1994;78:1–5.

(29) Yoshida M, Horinouchi S, Beppu T. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays 1995;17:423–30.

(30) Kwon HJ, Owa T, Hassig CA, Shimada J, Schreiber SL. Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase. Proc Natl Acad Sci U S A 1998;95:3356–61.

(31) Carducci M, Bowling MK, Eisenberger M, Sinibaldi V, Chen T, Nor D, et al. Phenylbutyrate (PB) for refractory solid tumors: phase I clinical and pharmacologic evaluation of intravenous and oral PB. Anticancer Res 1997;17:3972–3.

(32) Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, et al. A synthetic inhibitor of histone deacetylase, MS-27-275, with marked *in vivo* antitumor activity against human tumors. Proc Natl Acad Sci U S A 1999; 96:4592–7.

(33) Nakajima H, Kim YB, Terano H, Yoshida M, Horinouchi S. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res 1998;241:126–33.

(34) Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, et al. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A 1998;95:3003–7.

(35) Kim YB, Lee KH, Sugita K, Yoshida M, Horinouchi S. Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. Oncogene 1999;18:2461–70.

(36) Glick RD, Swendeman SL, Coffey DC, Rifkind RA, Marks PA, Richon VM, et al. Hybrid polar histone deacetylase inhibitor induces apoptosis and CD95/CD95 ligand expression in human neuroblastoma. Cancer Res 1999; 59:4392–9.

(37) Cohen LA, Amin S, Marks PA, Rifkind RA, Desai D, Richon VM. Chemoprevention of carcinogen-induced mammary tumorigenesis by the hybrid polar cytodifferentiation agent, suberanilohydroxamic acid (SAHA). Anticancer Res 1999;19:4999–5005.

(38) Butler LM, Higgins B, Fox WD, Agus DB, Cordon-Cardo C, Scher HJ, et al. Hybrid polar inhibitors of histone deacetylase suppress the growth of the CWR22 human prostate cancer xenograft. Proc Am Assoc Cancer Res 2000;41:abstract 289.

(39) Desai D, El-Bayoumy K, Amin S. Chemopreventive efficacy of suberanilohydroxamic acid (SAHA), a cytodifferentiating agent, against tobacco-specific nitrosamine 4-(methylnitros-amino)-1-(3-pyridyl)-1butanone (NNK)-induced lung tumorigenesis [abstract]. Proc Am Assoc Cancer Res 1999;40:abstract 2396.

(40) Qui L, Kelso MJ, Hansen C, West ML, Fairlie DP, Parsons PG. Antitumour activity *in vitro* and *in vivo* of selective differentiating agents containing hydroxamate. Br J Cancer 1999;80:1252–8.

(41) Kornberg RD. Chromatin structure: a repeating unit of histones and DNA. Science 1974;184:868–71.

(42) Kornberg RD, Lorch Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell 1999;98:285–94.

(43) Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 1997; 389:251–60.

(44) Davie JR. Covalent modifications of histones: expression from chromatin templates. Curr Opin Genet Dev 1998;8:173–8.

(45) Kouzarides T. Histone acetylases and deacetylases in cell proliferation. Curr Opin Genet Dev 1999;9:40–8.

(46) Bannister AJ, Kouzarides T. The CBP co-activator is a histone acetyltransferase. Nature 1996;384:641–3.

(47) Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996;87:953–9.

(48) Mizzen CA, Yang XJ, Kokubo T, Brownell JE, Bannister AJ, Owen-Hughes T, et al. The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. Cell 1996;87:1261–70.

(49) Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, et al. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 1997; 389:194–8.

(50) Siddique H, Zou JP, Rao VN, Reddy ES. The BRCA2 is a histone acetyltransferase. Oncogene 1998;16:2283–5.

(51) Waltzer L, Bienz M. *Drosophila* CBP represses the transcription factor TCF to antagonize Wingless signalling. Nature 1995;395:521–5.

(52) Taunton T, Hassig CA, Schreiber SL. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 1996:272:408–11.

(53) Emiliani S, Fischle W, Van Lint C, Al-Abed Y, Verdin E. Characterization of a human RPD3 ortholog, HDAC3. Proc Natl Acad Sci U S A 1998;95: 2795–800.

(54) Grozinger CM, Hassig CA, Schreiber SL. Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A 1999;96:4868–73.
(55) Verdel A, Khochbin S. Identification of a new family of higher eukaryotic histone deacetylases. Coordinate expression of differentiation-dependent chromatin modifiers. J Biol Chem 1999;274:2440–5.
(56) Fischle W, Emiliani S, Hendzel MJ, Nagase T, Nomura N, Voetler W, et al. A new family of human histone deacetylases related to *Saccharomyces cerevisiae* HDA1p. J Biol Chem 1999;274:11713–20
(57) Miska EA, Karlsson C, Langley E, Nielsen SJ, Pines J, Kouzarides T. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J 1999;18:5099–107.
(58) Kao HY, Downes M, Ordentlich P, Evans RM. Isolation of a novel deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev 2000;14:55–66.
(59) Hu E, Chen Z, Frederickson T, Zhu Y, Kirkpatrick R, Zhang GF, et al. Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. J Biol Chem 2000;275:254–64.
(60) Allfrey VG. Post synthetic modifications of histone: a mechanism for the control of chromosome structure by the modulation of histones—DNA interactions In: Li HJ, Eckhardt RA, editors. Chromatin and chromosome structure. New York (NY): Academic Press; 1977. p. 167–91.
(61) Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999;21:103–7.
(62) Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 1998;19:187–91.
(63) Bird AP, Wolffe AP. Methylation-induced repression—belts, braces, and chromatin. Cell 1999;99:451–4.
(64) Vignali M, Hassan AH, Neely KE, Workman JL. ATP-dependent chromatin-remodeling complexes. Mol Cell Biol 2000;20:1899–910.
(65) Tyler JK, Kadonaga JT. The "dark side" of chromatin remodeling: repressive effects of transcription. Cell 1999;99:443–6.
(66) Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 1997;90:595–606.
(67) Boyes J, Byfield P, Nakatani Y, Ogryzko V. Regulation of activity of the transcription factor GATA-1 by acetylation. Nature 1998;396:594–8.
(68) Imhof A, Yang XJ, Ogryzko VV, Nakatani Y, Wolffe AP, Ge H. Acetylation of general transcription factors by histone acetyltransferases. Curr Biol 1997;7:689–92.
(69) Luo RX, Postigo AA, Dean DC. Rb interacts with histone deacetylase to repress transcription. Cell 1998;92:463–73.
(70) Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 1998;391:597–601.
(71) Petrij F, Giles RH, Dauwerse HG, Saris JJ, Hennekam RC, Masuno M, et al. Rubinstein–Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature 1995;376:348–51.
(72) Sobulo OM, Borrow J, Tomek R, Reshmi S, Harden A, Schlegelberger B, et al. MLL is fused to CBP, a histone acetyltransferase, in therapy-related acute myeloid leukemia with a t(11;16)(q23;p13.3). Proc Natl Acad Sci U S A 1997;94:8732–7.
(73) Giles RH, Peters DJ, Breuning MH. Conjunction dysfunction: CBP/p300 in human disease. Trends Genet 1998;14:178–83.
(74) Warrell RP Jr, He LZ, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst 1998;90:1621–5.
(75) Novich S, Camacho L, Gallagher R, Chanel S, Ho R, Tolentino T, et al. Initial clinical evaluation of "transcription therapy" for cancer: all-*trans* retinoic acid plus phenylbutyrate. Blood 1999;94:Suppl 1, p61a.
(76) Dhordain P, Lin RJ, Quief S, Lantoine D, Kerckaert JP, Evans RM, et al. The LAZ3(BCL-6) oncoprotein recruits a SMRT/mSIN3A/histone deacetylase containing complex to mediate transcriptional repression. Nucleic Acids Res 1998;26:4645–51.
(77) Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci U S A 1998;95:10860–5.
(78) Darkin-Rattray SJ, Gurnett AM, Myers RW, Dulski PM, Crumley TM, Allocco JJ, et al. Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase. Proc Natl Acad Sci U S A 1996;93:13143–7.
(79) Breslow R, Jursic B, Yan ZF, Friedman E, Leng L, Ngo L, et al. Potent cytodifferentiating agents related to hexamethylenebisacetamide. Proc Natl Acad Sci U S A 1991;88:5542–6.
(80) Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, et al. Structures of a histone deacetylase homologue bound to TSA and SAHA. Nature 1999;401:188–93.
(81) Riggs MG, Whittaker RG, Neumann JR, Ingram VS. n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature 1997;268:462–4.
(82) Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular gene is changed in response to histone hyperacetylation. Gene Expr 1996;5:245–4.
(83) Xiao H, Hasegawa T, Isobe KI. Both Sp1 and Sp3 are responsible for p21 waf1 promoter activity induced by histone deacetylase inhibitor in NIH3T3 cells. J Cell Biochem 1999;73:291–302.
(84) Richon VM, Sandhoff TW, Rifkind RA, Marks PA. SAHA induced histone hyperacetylation of the $p21^{waf1}$ gene precedes transcription. Proc Am Assoc Cancer Res 2000;40:abstract 5131.

NOTES

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Exhibit 4

Editorial

Inhibitors of Histone Deacetylase Are Potentially Effective Anticancer Agents

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During the past decade, a number of HDAC[2] inhibitors have been identified that induce tumor cells in culture and in tumor-bearing animals to undergo growth arrest, differentiation, and/or apoptotic cell death (1). Acetylation and deacetylation of histones, part of the core proteins of nucleosomes, play an important role in the regulation of gene expression (2). There are two classes of enzymes involved in determining the degree of acetylation of histones, HATs, and HDACs. At least four groups of proteins with HAT activity and eight HDACs have been identified in mammalian cells (1–3). HDACs are generally found in association with large protein complexes that are involved in regulation of gene expression. HDACs may also regulate gene expression by deacetylating transcription factors, such as p53, and may participate in cell cycle regulation. It has been shown that RB (retinoblastoma protein) binding to the transcription factor E2F involves recruitment of HDAC1 or HDAC2. There are several reports that altered HAT or HDAC activity is associated with cancers.

HDAC Inhibitors

HDAC inhibitors that have been shown to arrest tumor cell growth belong to several chemical structural classes including: (*a*) hydroxamic acids, *e.g.*, TSA, whose activity is reported by Vigushin *et al.* (4) and a series of hybrid polar hydroxamic acid compounds, of which SAHA is a prototype, described by investigators at our laboratory (5); (*b*) short-chain fatty acids, *e.g.*, butyric acid; (*c*) cyclic tetrapeptides containing a AOE moiety, *e.g.*, trapoxin; (*d*) cyclic peptides not containing the AOE moiety, *e.g.*, FR901228 and apicidin; and (*e*) benzamides, *e.g.*, MS-27–275 (reviewed in Ref. 1).

The series of hydroxamic acid-based hybrid polar compounds inhibit HDACs activity at or below micromolar concentrations, both *in vitro* and *in vivo*. X-ray crystallographic analyses of a HDAC-like protein (HDLP), isolated from an anaerobic bacterium, showed that the catalytic site of the enzyme has a tubular pocket with a zinc-binding site at its base and two Asp-Histidine charge relay systems (6). The hydroxamic moiety of TSA and that of SAHA were shown to bind to the zinc at the base of the tubular pocket, and the carbon ring of these compounds projects out of the pocket onto the surface of the protein.

Antitumor Activity of HDAC Inhibitors *in Vitro* and *in Vivo*

HDAC inhibitors have been shown to cause growth arrest, differentiation, and or apoptotic cell death in a wide variety of transformed cells in culture, including neuroblastoma, melanoma, and leukemia, as well as cells from breast (Fig. 1), prostate, lung, ovarian, and colon cancers (reviewed in Ref. 1). Several HDAC inhibitors including the depsipeptide MS-27–275, oxamflatin, and the hydroxamic acid-based hybrid polar compounds, such as SAHA, have been shown to inhibit tumor growth in cancer-bearing animal models. These previous studies included the demonstration that the HDAC inhibitor, SAHA, inhibits methylnitrosourea-induced mammary breast carcinoma in rats (7), a finding similar to that reported with TSA by Vigushin *et al.* (4). Yoshida *et al.* (8) reported that TSA was a potent inhibitor of HDAC activity in both purified enzyme preparation and cells in culture. It is interesting to note that Qui *et al.* (9) reported that TSA did not inhibit the growth of human melanoma xenograft in nude mice, but the tumor growth was inhibited by azeloic bis-hydroxamide, structurally related to SAHA. In studies demonstrating that SAHA inhibits growth of xenograft human prostate tumors in nude mice, the HDAC inhibitor was shown to increase the accumulation of acetylated histone in tumor tissue, as well as, normal tissues (10). Furthermore, SAHA induced essentially complete inhibition of prostate cancer cell growth in these mice studies with little or no detectable toxicity. The accumulation of acetylated histones in peripheral mononuclear cells, as well as in tumor tissue, has been found in patients treated with as little as 75 mg/m^2 SAHA as a 2-h infusion. Assay for the accumulation of acetylated histones (1, 5) is useful as a biological marker of activity of the administered HDAC inhibition, as shown with the studies with TSA (4). SAHA and a second hybrid polar hydroxamic acid-based HDAC inhibitor, pyroxamide, are in Phase I clinical trials.

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[2] The abbreviations used are: HDAC, histone deacetylase; HAT, histone acetyltransferase; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; AOE, 2-amino-8-oxo-9,10 epoxy-decanoyl.

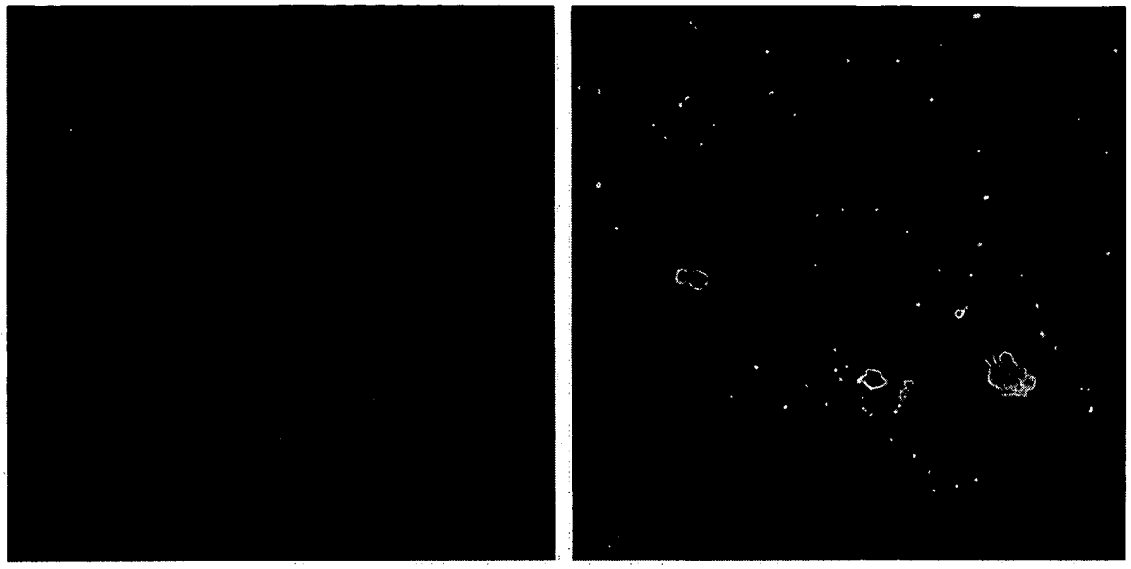

Fig. 1 Effect of SAHA on human breast adenocarcinoma cells MCF-7 in culture. *Left*, MCF-7 cells cultured with diluent control (*DMSO*); *right*, MCF-7 cells cultured with 5 μM SAHA for 48 h. *Red*, rodamine-labeled anti-milk fat membrane globule; *green*, fluorescent-labeled anti-milk fat globule; *blue*, DNA stained with bis-benzamide.[3]

References

1. Marks, P. A., Richon, V. M., and Rifkind, R. A. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J. Natl. Cancer Inst. (Bethesda), *92:* 1210–1215, 2000.

2. Grunstein, M. Histone acetylation and chromatin structure and transcription. Nature (Lond.), *389:* 349–352, 1997.

3. Kouzarides, T. Histone acetylases and deacetylases in cell proliferation. Curr. Opin. Genet. Dev., *9:* 40–48, 1999.

4. Vigushin, D. M., Ali, S., Pace, P. E., Mirsaidi, N., Ito, K., Adcock, I., and Coombes, R. C. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer *in vivo*. Clin. Cancer Res., *7:* in press, 2001.

5. Richon, V. M., Emiliani, S., Verdin, E., Webb, Y., Breslow, R., Rifkind, R. A., and Marks, P. A. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc. Natl. Acad. Sci, USA, *95:* 3003–3007, 1998.

6. Finnin, M. S., Donigian, J. R., Cohen, A. Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature (Lond.), *401:* 188–193, 1999.

7. Cohen, L. A., Amin, S., Marks, P. A., Rifkind, R. A., Desai, D., and Richon, V. M. Chemoprevention of carcinogen-induced mammary tumorigenesis by the hybrid polar cytodifferentiation agent syberanilohydroxamic acid (SAHA). Anticancer Res., *19:* 4999–5005, 1999.

8. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by Trichostatin A. J. Biol. Chem., *265:* 17174–17179, 1990.

9. Qui, L., Kelson, M. J., Hanse, C., West, M. L., Fairlie, D. P., and Parsons, P. G. Anti-tumor activity *in vitro* and *in vivo* of selective differentiating agents containing hydroxamide. Br. J. Cancer, *80:* 1252–1258, 1990.

10. Butler, L. M., Agus, D., Scher, H. I., Higgins, B., Rose, A., Cordon-Cardo, C., Thaler, H. T., Rifkind, R. A., Marks, P. A., and Richon, V. M. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells *in vitro* and *in vivo*. Cancer Res., *60:* 5165–5170, 2000.

[3] Figure was kindly provided by P. N. Munster, T. Torso-Sandoval, N. Rosen, R. A. Rifkind, P. A. Marks, and V. M. Richon. Suberoylanilide hydroxamic acid induces differentiation in mammary epithelial carcinoma cells, submitted for publication.

Exhibit 5

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Chemistry

Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase

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Contributed by Stuart L. Schreiber, December 31, 1997

ABSTRACT **Depudecin is a fungal metabolite that reverts the rounded phenotype of NIH 3T3 fibroblasts transformed with v-*ras* and v-*src* oncogenes to the flattened phenotype of the nontransformed parental cells. The mechanism of detransformation induced by this agent had not been determined. Here, we demonstrate that depudecin inhibits histone deacetylase (HDAC) activity effectively both *in vivo* and *in vitro*. Depudecin induces similar morphological reversion in v-*ras* transformed NIH 3T3 cells as do other naturally occurring HDAC inhibitors such as trichostatin A or trapoxin. It competitively inhibits the binding of [^{3}H]trapoxin *in vitro* and the nuclear binding of a trapoxin–coumarin fluorophore *in vivo*, suggesting that depudecin shares a nuclear binding protein and site on that protein with trapoxin. Furthermore, depudecin induces hyperacetylation of histones in a dose-dependent manner and at concentrations comparable with that required for detransformation. An *in vitro* histone deacetylase assay, using purified recombinant HDAC1, reveals that depudecin inhibits 50% of the enzyme activity at a concentration of 4.7 μM. These results demonstrate that depudecin is a novel HDAC inhibitor and suggest that its ability to induce morphological reversion of transformed cells is the result of its HDAC inhibitory activity.**

The shape and cytoskeletal architecture of cells are often dramatically altered as a consequence of malignant transformation (1). One of the most characteristic cytoskeletal changes in tumor cells is the loss of actin stress fibers, suggesting that the actin-containing microfilament system is a critical target during tumorigenesis (2). Agents that reorganize these fibers, reverting the transformed morphology to a normal one, can be used to probe growth factor-mediated signaling pathways leading to actin stress fiber formation, and have potential as therapeutics for treatment of cancer.

Several natural products have been isolated by screening for detransforming activity using oncogene-transformed cells. Examples include trapoxin (3, 4), trichostatin A (5), herbimycin A (6), radicicol (7), and azatyrosine (8). These compounds are able to revert the morphological changes seen following the transformation of cells in culture with v-*sis*, v-*src*, or v-*ras* oncogenes and in cells derived from tumors. For instance, trichostatin A and trapoxin are known to revert v-*sis* and v-*ras* oncogene-transformed cells as well as human tumor cells such as HeLa and T24 cells (9, 10). It has been unclear how these compounds are able to show a broad spectrum of detransforming activity in tumor cells. Histone deacetylases (HDACs) were considered likely molecular targets of these agents (11), and, recently, human histone deacetylase (HDAC1) was purified and cloned by using a trapoxin-based affinity matrix purification (12). Accumulating evidence suggests that the acetylation and deacetylation of histones play significant roles

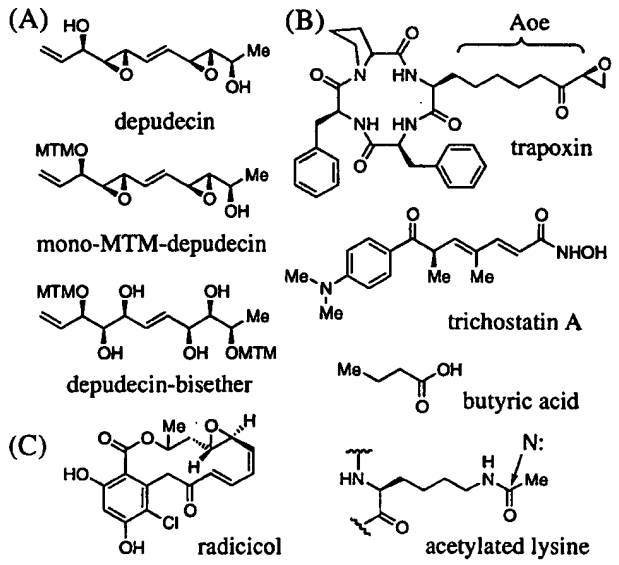

FIG. 1. The chemical structures of depudecin and other compounds discussed in text. (*A*) Depudecin and its inactive derivatives, mono-MTM-depudecin and depudecin-bisether. (*B*) HDAC inhibitors trapoxin, trichostatin, and butyric acid. (*C*) Radicicol. Trapoxin and other cyclic tetrapeptide inhibitors contain the functionally important amino acid (2*S*,9*S*)-2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe). This side chain is approximately isosteric to that of ε-*N*-acetylated lysine residues in histone proteins and may form a bond to a nucleophilic residue in the enzyme active site.

in the regulation of transcription in eukaryotic cells (13–16). The broad spectrum of detransforming activities displayed by HDAC inhibitors implies a role for HDACs in stress fiber formation and cell growth control (9, 11). HDACs may repress the transcription of anti-tumor genes whose products induce actin stress fiber reorganization and arrest cell proliferation.

Trichostatin A and trapoxin are the only detransforming agents known to target HDACs. For example, radicicol, shown here not to inhibit HDAC enzymatic activity or trapoxin binding to HDACs, is thought to mediate its actions by inhibiting Src tyrosine kinase activity (7). Depudecin (17) is a structurally novel natural product whose molecular target had not previously been defined (18). It was discovered in the culture broth of the fungus *Alternaria brassicicola* by assaying for the morphological detransformation of NIH 3T3 cells doubly transfected with v-*ras* and v-*src* oncogenes. Depudecin does not suppress the expression of the *ras* gene, suggesting that its mode of action does not relate directly to Ras function

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Abbreviations: HDAC, histone deacetylase; AUT, acid/urea/Triton X-100.

A commentary on this article begins on page 3335.

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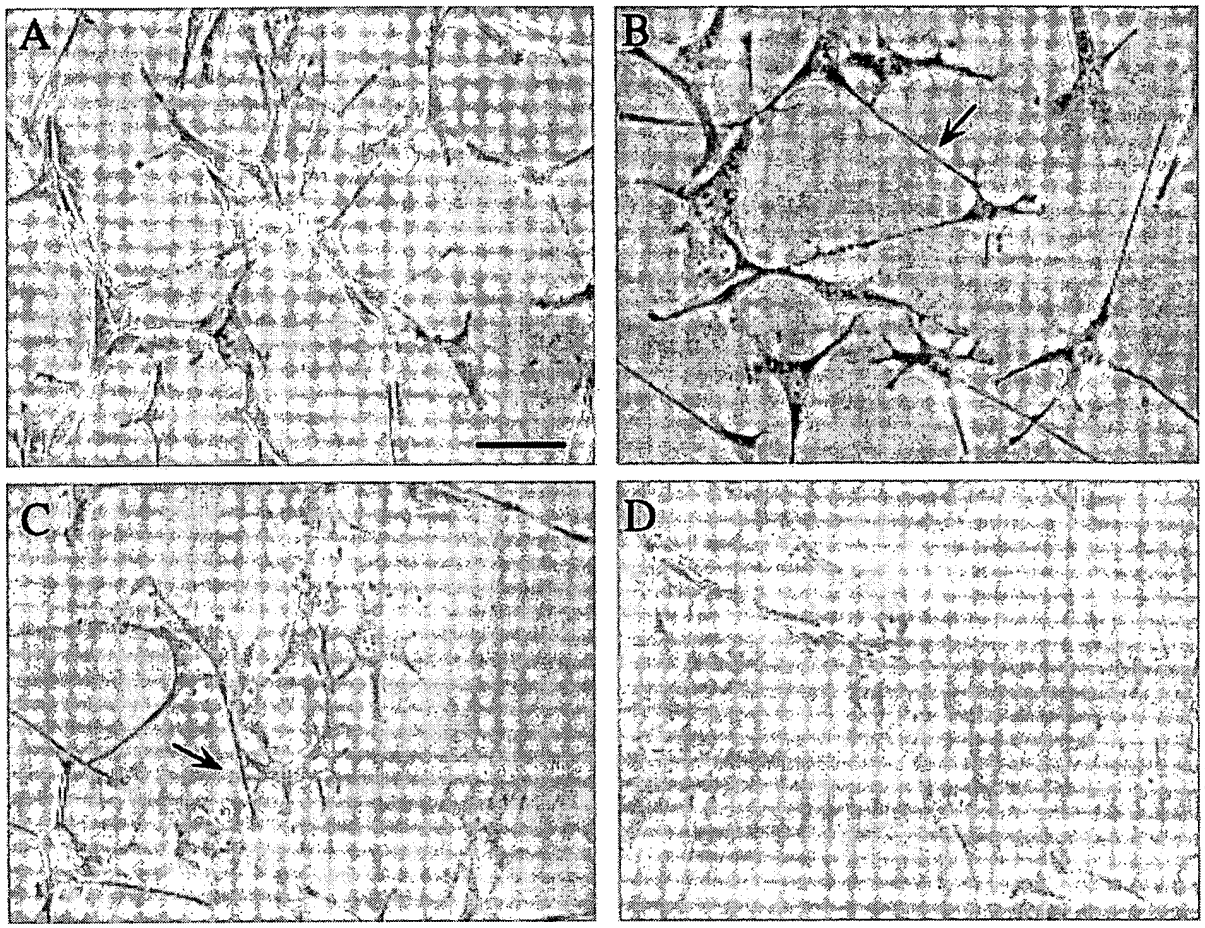

FIG. 2. The morphological change in v-*ras*-transformed NIH 3T3 cells by detransforming agents. Exponentially growing cells were treated for 24 h with test agents, and photographs were taken under a phase-contrast microscope. (*A*) Control v-*ras* transformed NIH 3T3 cells. (*B*) Cells treated with 4.7 μM synthetic depudecin. (*C*) Cells treated with 1.5 μM trichostatin A. (*D*) cells treated with 3.4 μM radicicol. The characteristic elongated cells having filamentous protrusions induced by depudecin and trichostatin A are indicated by the arrow. (Bar = 50 μm.)

(19). It also shows a spectrum of detransforming activity, both in v-*raf* transformed cells and in a human osteosarcoma cell line, MG63 (18).

In this study, we demonstrate that depudecin inhibits histone deacetylase activity both *in vivo* and *in vitro*. Depudecin represents the fourth structural class of molecules (see Fig. 1) having this activity. Like the other three, its inhibitory activity can be rationalized in terms of its unique chemical structure and the anticipated structural features of the substrate binding and catalytic sites on HDAC enzymes. Like the actin and tubulin proteins, which are liganded by many different microbial products having diverse chemical structures, HDACs seem to have been targeted frequently by microbial biosynthetic pathways.

MATERIALS AND METHODS

Materials. Depudecin and its inactive derivatives used in this study were prepared by total synthesis according to the method of Shimada *et al.* (18). [^{3}H]Trapoxin was prepared by total synthesis as described by Taunton *et al.* (20). Trichostatin A was purchased from Biomol (Plymouth Meeting, PA), and radicicol was provided generously as a gift by Professor W. C. Taylor (University of Sydney). DMEM, RPMI media, fetal calf serum, glutamine, and penicillin-streptomycin were purchased from GIBCO/BRL.

Synthesis of a Trapoxin–Coumarin Fluorophore. An allyloxycarbonyl-protected, lysine variant of trapoxin, previously named "K-trap," was prepared as described by Taunton *et al.* (20). The allyloxycarbonyl group was removed from K-trap by brief treatment with 10 mol% of tris(dibenzylideneacetone) dipalladium chloroform and 40 mol% of triphenylphosphine in the presence of dimedone (5 equivalent). The crude amine was purified immediately by reverse phase HPLC, lyophilized, and coupled to *N*-hydroxysuccinimide ester of a coumarin dye (Molecular Probes) in the presence of excess *N,N*-diisopropylethylamine. The reaction mixture was treated with saturated $NaHCO_3$, extracted with EtOAc, dried by filtration through anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by small pipet column chromatography to afford a trapoxin–coumarin conjugate, named "K-trap–coumarin dye" in Fig. 4 (molecular weight 898.07) in 65% combined yield for two steps. Its molecular weight and chemical structure were confirmed using fast atom bombardment-MS and ^{1}H-NMR.

Cells and Cell Culture Conditions. v-*ras* transformed NIH 3T3 cells were kindly supplied by Professor R. L. Erickson (Molecular and Cellular Biology, Harvard University), and HL60 and COS cells were purchased from ATCC. These cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in DMEM (v-*ras* NIH 3T3 and COS) or RPMI (HL60) medium supplemented with 10% fetal calf serum, glutamine, and penicillin-streptomycin.

Cell Staining with a Trapoxin–Coumarin Fluorophore. To obtain evidence of *in vivo* binding of depudecin to nuclear HDACs, cells were stained with the trapoxin–coumarin flu-

orophore in analogy to the method of Shimada *et al.* (18). Briefly, asynchronous cells were cultured on a slide coverslip for 18 h. After treatment with depudecin or other inhibitors for an additional 24 h, cells were fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature. Following three times washing with PBS, cells were treated with 0.1% Triton X-100 in PBS for 5 min to increase cell permeability. Next, the cells were treated with the trapoxin–coumarin fluorophore (5 μg) for 20 min, and after washing three times with PBS, the cells were mounted on a slide with an antifader (DABCO, 1, 4-diazabicyclo[2.2.2]-octane, Aldrich) in 50% glycerol. Cells were inspected by conventional fluorescence microscopy (Leitz Wetzlar, Germany).

[^{3}H]Trapoxin Binding Assay. Soluble extracts for binding studies were prepared by homogenizing 1×10^8 v-*ras* NIH 3T3 or HL60 cells in 50 mM Tris-HCl (pH 7.5) with 100 mM NaCl and 10% glycerol. The resulting homogenate was centrifuged in a microcentrifuge at maximum speed (15,000 rpm) for 15 min, and the supernatant was used directly. For the competition binding assay with [^{3}H]trapoxin, test compounds were preincubated with the supernatant (200 μg/100 μl) for 1 h at 25°C, and then 100 nM [^{3}H]trapoxin was added for 30 min at 25°C. Free radioactivity was removed by precipitation with 1% charcoal solution in 10% BSA, and the binding activity was determined by scintillation counting of the supernatant.

Histone Hyperacetylation Assay. Histones were extracted from v-*ras* NIH 3T3 or HL60 cells (2×10^7 cells) that had been treated with test compounds for 6 h at 37°C as described by Yoshida *et al.* (21). Acid urea gel electrophoresis was used for the detection of acetylated histone molecules. Slab gels were run at 12 mA for 10 h at room temperature as described by Panyim and Chalkley (22) using a slab gel.

Histone Deacetylase Assays. *In vitro* histone deacetylase activity was assayed as described previously with either 100 μl of crude cell extract (1×10^7 cells) or 25 ng of recombinant FLAG epitope-tagged HDAC1 (HDAC1-F) for 3 h at 37°C (refs. 12 and 23; C.A.H., J. K. Tong, T.O., D. E. Ayer, and S.L.S., unpublished results). Pretreatment of crude or HDAC1-F enzymes with test compounds was performed for 30 min at 4°C prior to addition of the [^{3}H]acetyl histone substrate that was acetylated with [^{3}H]acetate (0.5 Ci/mmol, New England Nuclear) by an *in vivo* incorporation method (21). The reaction was quenched with 1 M HCl and 0.16 M acetic acid (50 μl). Released [^{3}H]acetate was extracted with 600 μl of ethyl acetate and quantified by scintillation counting.

RESULTS

Induction of Morphological Change in v-*ras* NIH 3T3 Cells by Depudecin. v-*ras* NIH 3T3 is a cell line that is known to revert from a spindle-like, transformed to a normal morphology following treatment with depudecin, trichostatin A, or radicicol. We first compared the effects of these agents on cell morphology. After treatment for 24 h, the cells showed pronounced and characteristic morphological alterations depending on the agent (Fig. 2). Generally, the spindle-like morphology of v-*ras* NIH 3T3 cells was changed to a flattened morphology by all of these compounds, as reported previously (9, 18, 24). However, cells treated with depudecin and trichostatin A produced characteristic elongated cells with filamentous protrusions (noted by arrows in Fig. 2), whereas the tyrosine kinase inhibitor radicicol did not. Trapoxin, another HDAC inhibitor, induced morphologic alterations reminiscent of those induced by depudecin and trichostatin A (data not shown), suggesting that the detransforming activity of depudecin may also be due to the inhibition of HDAC enzymes.

Depudecin Binds Competitively to a [^{3}H]Trapoxin-Binding Protein. To explore HDAC inhibition as the potential mode of action of depudecin in detransformation, a binding assay was carried out to see whether depudecin shares a binding protein with known HDAC inhibitors. [^{3}H]Trapoxin was chosen as the radioligand because it shows a high specific binding to HDACs (20) (Fig. 3). Depudecin, like trapoxin, also effectively inhibited [^{3}H]trapoxin binding in a dose-dependent manner. However, radicicol was largely ineffective as a competitive inhibitor of [^{3}H]trapoxin binding. These results indicate that depudecin shares common target proteins with trapoxin, likely the HDAC enzymes, and that radicicol does not.

Depudecin Prevents Appearance of Fluorescence in the Nucleus Induced by a Trapoxin–Coumarin Dye. To determine whether depudecin binds to HDACs in v-*ras* NIH 3T3 and COS cells, we synthesized a fluorescent variant of trapoxin, named K-trap–coumarin dye (Fig. 4). This reagent inhibited HDAC activity at a concentration similar to that of K-trap (100 nM) both *in vitro* and *in vivo*, suggesting that K-trap–coumarin dye binds HDACs and should therefore be competed by the pretreatment of other HDAC inhibitors. As shown in Fig. 4, K-trap–coumarin dye stains the nuclei (blue color), whereas coumarin dye control does not show a significant signal (Fig. 4 *A* and *B*, *C* and *D*, respectively). This result provides evidence for the expected nuclear localization of HDAC proteins. When cells were pretreated with depudecin or trapoxin, similar morphological changes (i.e., spread and elongated protrusion morphology) were observed, but the fluorescence signal was weaker and distributed throughout the cell (Fig. 4 *E* and *F*, *G* and *H*, respectively). This result suggests that depudecin competes with K-trap–coumarin dye for binding to HDACs in cells, as does trapoxin. Interestingly, cells treated with radicicol show an altered (flattened) morphology, but the K-trap–coumarin dye stains the nuclei to comparable levels as the control, suggesting that radicicol induces a morphological change by a mechanism not involving HDAC binding (Fig. 4 *I* and *J*). Depudecin-bisether, an inactive depudecin derivative, does not change the morphology of cells nor inhibit the K-trap–coumarin dye from staining the nuclei (data not shown).

Depudecin Induces Histone Hyperacetylation *in Vivo*. We next investigated whether depudecin can inhibit histone

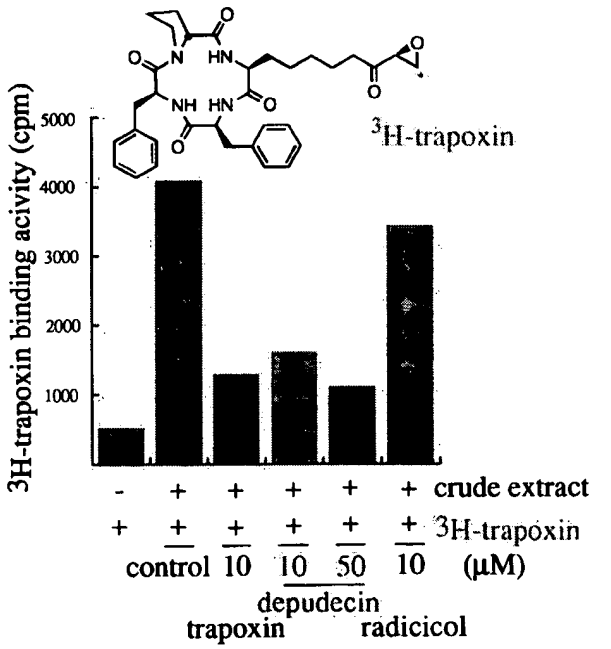

FIG. 3. The effect of depudecin and other detransforming agents on [^{3}H]trapoxin binding with crude lysate of v-*ras* transformed NIH 3T3 cells. The assay was carried out as described in *Material and Methods*. A 100-fold excess amount of cold compounds was preincubated with crude lysate for 30 min at 25°C in the competition assay.

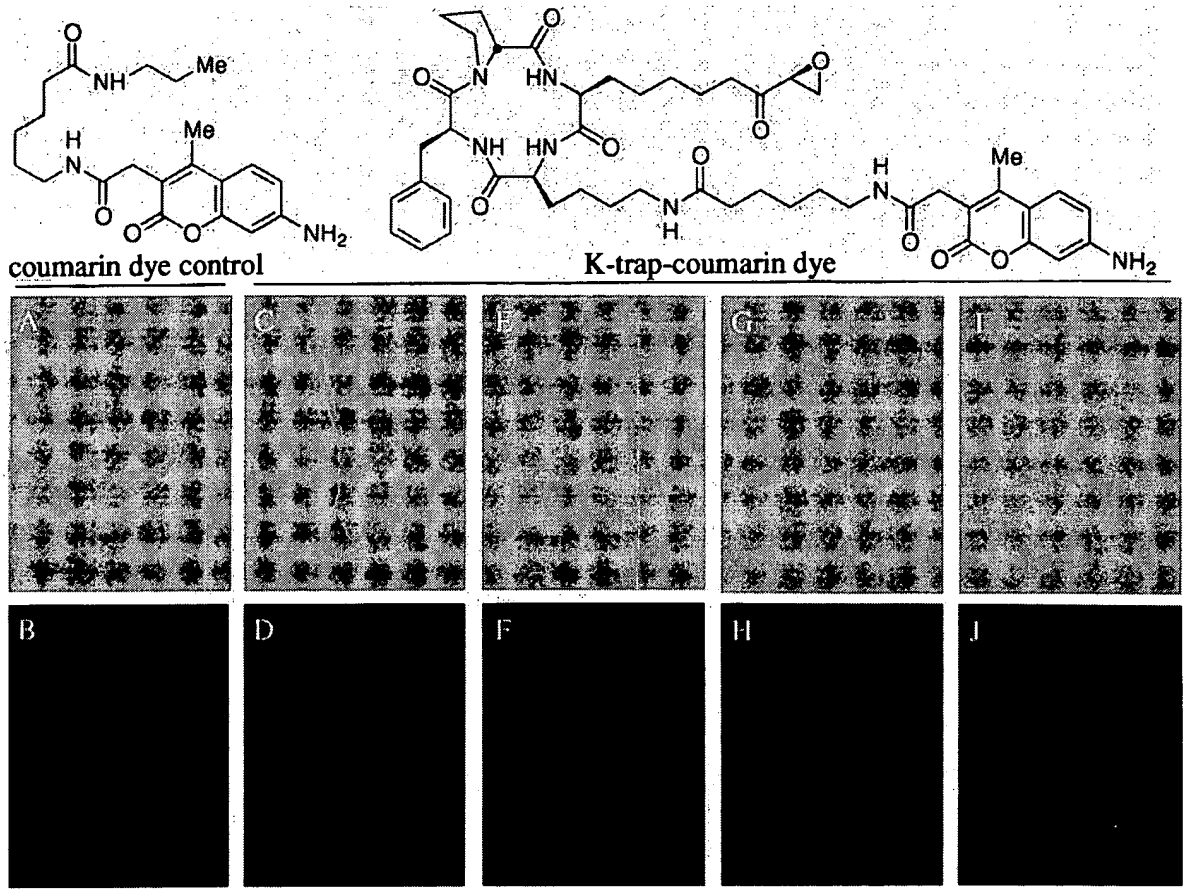

FIG. 4. Visualization of HDACs and HDAC binding in the nucleus of v-*ras* NIH 3T3 cells using a competition binding assay involving K-trap–coumarin dye and various detransformation agents. The staining was carried out as described in *Material and Methods*. Upper and lower photos were taken using phase-contrast and fluorescence microscopy, respectively. Coumarin dye excites at 345 nm with blue light. (*A* and *B*) Coumarin dye control. (*C* and *D*) K-trap–coumarin dye control. (*E* and *F*) K-trap–coumarin dye in cells pretreated with 23.5 μM depudecin. (*G* and *H*) K-trap–coumarin dye in cells pretreated with 0.1 μM trapoxin. (*I* and *J*) K-trap–coumarin dye in cells pretreated with 3.4 μM radicicol.

deacetylase activity in v-*ras* NIH 3T3 cells. It is known that inhibition of this activity by trichostatin A (21), trapoxin (10), and sodium butyrate (25) results in the accumulation of hyperacetylated histone species. This effect can be visualized by acid/urea/TritonX-100 (AUT) gel electrophoresis of histones extracted from inhibitor-treated cells. Increased acetylation results in a decreased mobility of histones in AUT gel electrophoresis and the appearance of multiple bands corresponding to histones with 1, 2, 3, and 4 acetylated residues. As shown in Fig. 5, depudecin induces hyperacetylation of histones (especially histone H4) in a dose-dependent manner. Trichostatin A, but not radicicol, also strongly induces hyperacetylation of histones. We had previously reported that mono-methylthiomethyl (MTM)-depudecin and depudecin-bisether, two inactive depudecin derivatives, did not induce detransformation in v-*ras* NIH 3T3 cells (18). These inactive depudecin derivatives do not induce accumulation of acetylated histones (Fig. 5, lanes 4 and 5). These results demonstrate that depudecin inhibits HDAC activity *in vivo* and suggest that depudecin's detransforming activity may be manifested via HDAC inhibition.

Depudecin Inhibits HDAC Activity *in Vitro*. Next, we confirmed the inhibitory activity of depudecin on purified HDACs *in vitro*. First, we used an extract from HL60 cells as a source of HDACs. Extracts were incubated with several detransforming agents and examined for HDAC activity using ^{3}H-acetylated histones as substrate. Depudecin inhibited the HDAC activity in a dose-dependent manner. Trichostatin A also showed strong inhibition of the enzyme, whereas radicicol did not (Fig. 6*A*). We next investigated the effect of depudecin on purified FLAG epitope-tagged HDAC1 (HDAC1-F) expressed in Sf9 insect cells using a baculovirus vector (23). As shown at Fig. 6*B*, depudecin inhibited the purified enzyme activity to levels comparable with the known HDAC inhibitors, trichostatin A, trapoxin, and chlamydocin (26). In contrast, radicicol had a negligible effect. Higher concentration (47 μM) of the inactive depudecin-bisether did reduce activity of pure HDAC1-F but not HDAC activity of an extract (data not shown). The IC_{50} value of depudecin in this assay (4.7 μM) is comparable with that determined in the detransforming assay (4.7–47 μM) (19).

DISCUSSION

Depudecin is a natural product having a highly unusual structure. Its 11-carbon chain contains two epoxides and six stereogenic centers. Until now, the mechanism of its ability to induce actin stress fiber formation in cells was unknown. In part by synthesizing a fluorescent HDAC ligand and using it as a visual reporter of nuclear HDAC binding, the present study demonstrates that depudecin inhibits HDAC activity both *in vitro* and *in vivo*. Because other, structurally unrelated HDAC inhibitors have similar effects on cells, including the ability to induce actin stress fibers, the results suggest that HDAC enzymes are the biologically relevant target proteins of depudecin. However, the present study also shows that at least one other inducer of actin stress fiber formation, radicicol, does not operate through an HDAC mechanism. Depudecin

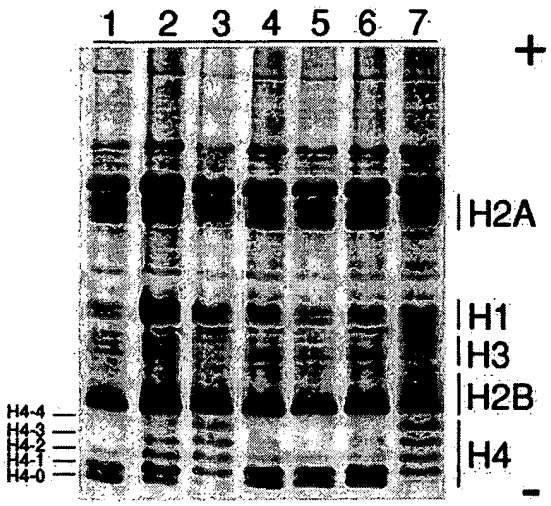

FIG. 5. The effect of depudecin and other detransforming agents on histone acetylation in v-*ras* transformed NIH 3T3 cells. Lane 1, control, treatment of v-*ras* transformed NIH 3T3 cells for 6 h with vehicle only; lane 2, 4.7 μM depudecin; lane 3, 23.5 μM depudecin; lane 4, 47 μM mono-MTM-depudecin; lane 5, 47 μM depudecin-bisether; lane 6, 3.4 μM radicicol; lane 7, 1.5 μM trichostatin A. Histones were extracted from the cells and visualized on AUT gel by Coomassie brilliant blue staining. Acetylated histone species have slower rates of migration relative to nonacetylated histones (H4-0, -1, -2, -3, -4).

and its derivatives should be useful tools to explore the role of HDACs and histone acetyltransferases (27) in the regulation of chromatin structure and gene transcription in eukaryotes (23, 28–31).

The molecular basis for the downstream effects of HDAC inhibition by depudecin remains to be determined. Depudecin induces not only morphological changes but also cell cycle arrest and cellular differentiation in many mammalian cell lines (H.J.K. and S.L.S., unpublished results), as do other HDAC inhibitors. The detransforming activity of depudecin is suppressed by actinomycin D and cycloheximide, suggesting that both mRNA synthesis and *de novo* protein synthesis are required for depudecin-mediated detransforming activity (18). HDAC inhibitors result in increased levels of gelsolin, a Ca^{2+}-dependent actin filament-severing and capping protein, likely by derepression of the gelsolin gene (9, 32). That this effect is relevant to the observed morphological changes is supported by the observation that HDAC inhibitor-induced morphologic changes are suppressed following microinjection of anti-gelsolin antibodies (33).

Several HDAC inhibitors have been used to explore the function of HDACs in cells. The earliest identified HDAC inhibitor, butyric acid, inhibits HDAC enzymatic activity at millimolar concentration, leading to concerns that this agent induces additional effects unrelated to histone hyperacetylation. The potency and apparent specificity of the natural product inhibitors, products of natural selection, largely alleviate these concerns. That nature has produced a wide variety of structures from a variety of sources for the purpose of HDAC inhibition suggests that these enzymes play essential and conserved functions. Two other essential proteins that have been targeted by this form of natural selection are actin and tubulin.

The mechanism of HDAC inhibition by depudecin may be related to that of the other natural inhibitors, especially that of trapoxin. Each of these compounds has an acyclic chain presumed to mimic the side chain of an ε-*N*-acetylated lysine residue. At the termini of these chains are different chemical moieties that likely interact with key catalytic elements in the

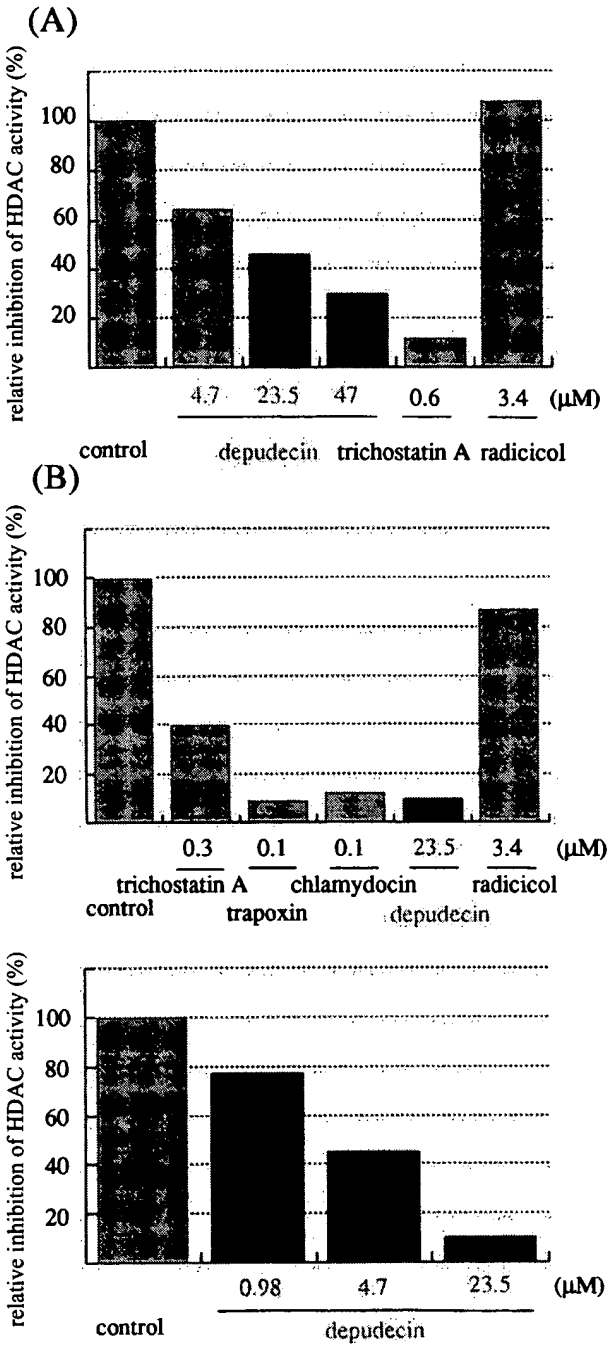

FIG. 6. The effect of depudecin and other detransforming agents on histone deacetylase *in vitro*. The assay was carried out as described in *Materials and Methods*. (*A*) HDAC assay using crude lysate as the source of enzyme. (*B*) HDAC assay using purified recombinant HDAC1-F.

enzyme active sites. The carboxylic and hydroxamic acid groups of butyrate and trichostatin most likely ligand a catalytic metal ion. The epoxide groups in the depudecin, trapoxin, and trapoxin-related natural products (e.g., chlamydocin) likely bond covalently with nucleophilic groups in the active sites. The selectivity of these inhibitors toward different HDAC family members is not currently known (12, 34, 35).

Depudecin and other HDAC inhibitors have potential as therapeutic agents. Apicidin, a cyclic peptide related to trapoxin, has been shown to exhibit potent antiprotozoal activity by

inhibition of HDACs in parasites (36). Depudecin has been shown to exhibit anti-angiogenesis activity both *in vitro* and *in vivo* (37), suggesting that specific inhibition of HDACs in endothelial cells could have therapeutic value. HDAC inhibitors arrest the cell cycle and revert the transformed morphology of p53-deficient cell lines HL60, HeLa, Saos-2, and of SV40-transformed cells (11), suggesting a strategy for treatment of tumors deficient in p53 function (38). Future investigations of HDAC function and HDAC-related therapies will benefit from variants of the natural inhibitors showing specificity for individual HDAC family members and for HDACs from specific organisms. Their structures, now including that of depudecin, combined with new methods in small molecule synthesis and screening, will provide a useful guide to such research efforts.

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1. Wahrman, M. Z., Gagnier, S. E., Kobrin, D. R., Higgins, P. J. & Augenlicht, L. H. (1985) *Tumor Biol.* **6**, 41–56.
2. Wang, Y.-I. & Goldberg, A. R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4065–4069.
3. Itazaki, H., Nagashima, K., Sugita, K., Yoshida, H., Kawamura, Y., Yasuda, Y., Matsumoto, K., Ishii, K., Uotani, N., Nakai, H., Terui, A., Yoshimatsu, S., Ikenishi, Y. & Nakagawa, Y. (1990) *J. Antibiot.* **43**, 1524–1532.
4. Yoshida, H. & Sugita, K. (1992) *Jpn. J. Cancer Res.* **83**, 324–328.
5. Sugita, K., Koizumi, K. & Yoshida, H. (1992) *Cancer Res.* **52**, 168–172.
6. Uehara, Y., Hori, M., Takeuchi, O. & Umezawa, H. (1986) *Mol. Cell. Biol.* **6**, 2198–2206.
7. Kwon, H. J., Yoshida, M., Fukui, Y., Horinouchi, S. & Beppu, T. (1992) *Cancer Res.* **52**, 6926–6930.
8. Shindo, O. N., Makabe, O., Nagahara, H. & Nishimura, S. (1990) *Basic Life Sci.* **52**, 309–312.
9. Hoshikawa, Y., Kwon, H. J., Yoshida, M., Horinouchi, S. & Beppu, T. (1994) *Exp. Cell Res.* **214**, 189–197.
10. Kijima, M., Yoshida, M., Sugita, K., Horinouchi, S. & Beppu, T. (1993) *J. Biol. Chem.* **268**, 22429–22435.
11. Yoshida, M., Horinouchi, S. & Beppu, T. (1995) *BioEssays* **17**, 423–430.
12. Taunton, J., Hassig, C. A. & Schreiber, S. L. (1996) *Science* **272**, 408–411.
13. Grunstein, M. (1997) *Nature* **389**, 349–352.
14. Loidl, P. (1994) *Chromosoma* **103**, 441–449.
15. Pazin, M. & Kadonaga, J. T. (1997) *Cell* **89**, 325–328.
16. Wolffe, A. P. (1996) *Science* **272**, 371–372.
17. Matsumoto, M., Matsutani, S., Sugita, K., Yoshida, H., Hayashi, F., Terui, Y., Nakai, H., Uotani, N., Kawamura, Y., Matsumoto, K., Shoji, J. & Yoshida, T. (1992) *J. Antibiot.* **45**, 879–885.
18. Shimada, J., Kwon, H. J., Sawamura, M. & Schreiber, S. L. (1995) *Chem. Biol.* **2**, 221–228.
19. Sugita, K., Yoshida, H., Matsumoto, M. & Matsutani, S. (1992) *Biochem. Biophys. Res. Commun.* **182**, 379–387.
20. Taunton, J., Collins, J. L. & Schreiber, S. L. (1996) *J. Am. Chem. Soc.* **118**, 10412–10422.
21. Yoshida, M., Kijima, M., Akita, M. & Beppu, T. (1990) *J. Biol. Chem.* **265**, 17174–17179.
22. Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346.
23. Hassig, C. A., Fleischer, T. C., Billin, A. N., Schreiber, S. L. & Ayer, D. E. (1997) *Cell* **89**, 341–347.
24. Kwon, H. J., Yoshida, M., Muroya, K., Hattori, S., Nishida, E., Beppu, T. & Horinouchi, S. (1995) *J. Biochem.* **118**, 221–228.
25. Riggs, M. G., Whittaker, R. G., Neumann, J. R. & Ingram, V. R. (1977) *Nature (London)* **268**, 462–464.
26. Shute, R. E., Dunlap, B. & Rich, D. J. (1987) *J. Med. Chem.* **30**, 71–78.
27. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. & Allis, C. D. (1996) *Cell* **84**, 843–851.
28. Durrin, L. K., Mann, R. K., Kayne, P. S. & Grunstein, M. (1991) *Cell* **65**, 1023–1031.
29. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W.-M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K. & Rosenfeld, M. G. (1997) *Nature (London)* **387**, 43–48.
30. Laherty, C. D., Yang, W.-M., Sun, J.-M., Davie, J. R., Seto, E. & Eisenman, R. N. (1997) *Cell* **89**, 349–356.
31. Yang, X.-J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. & Nakatani, Y. (1996) *Nature (London)* **382**, 319–324.
32. Tanaka, M., L., M., Ogiso, Y., Fujita, H., Moriya, S., Fruuchi, K., Harabayashi, T., Shinohara, N., Koyanagi, T. & Kuzumaki, N. (1995) *Cancer Res.* **55**, 3228–3232.
33. Kwon, H. J. (1995) Ph.D. thesis (University of Tokyo).
34. Yang, W.-M., Inouye, C., Zeng, Y., Bearss, D. & Seto, E. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 12845–12850.
35. Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M. & Grunstein, M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14503–14508.
36. Darkin-Rattray, S. J., Gurnett, A. M., Myers, R. W., Dulski, P. M., Crumley, K. M., Allocco, J. J., Cannova, C., Meinke, P. T., Colletti, S. L., Bednarek, M. A., Singh, S. B., Goetz, M. A., Dombrowski, A. W., Polishook, J. D. & Schimatz, D. M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13143–13147.
37. Oikawa, T., Onozawa, C., Inose, M. & Sasaki, M. (1995) *Biol. Pharm. Bull.* **18**, 1305–1307.
38. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. & Jacks, T. (1993) *Nature (London)* **362**, 847–849.

Exhibit 6

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Genetics

Trichostatin A causes selective loss of DNA methylation in *Neurospora*

(histone acetylation/silencing/epigenetics/5-azacytidine/deacetylase)

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ABSTRACT **Both DNA methylation and hypoacetylation of core histones are frequently associated with repression of gene expression. Possible connections between these processes were investigated by taking advantage of genes controlled by methylation in *Neurospora crassa*. Trichostatin A (TSA), a potent inhibitor of histone deacetylase, derepressed a copy of *hph* that was repressed by DNA methylation which resulted from repeat-induced point mutation (RIP) acting on sequences flanking *hph*. Derepression by TSA was comparable to derepression by the inhibitor of DNA methylation, 5-azacytidine. TSA treatment also repressed an allele of *am* whose expression depends on methylation of an adjacent transposon, *Tad*. DNA methylation in the *hph* and *Tad/am* regions was greatly reduced by TSA treatment. TSA also caused hypomethylation of other methylated alleles of *am* generated by RIP. In contrast, TSA did not affect methylation of several other methylated genomic sequences examined, including the nucleolar rDNA and the inactivated transposon *Punt*^{RIP1}. Several possible models are discussed for the observed selective demethylation induced by TSA. The implication that acetylation of chromatin proteins can directly or indirectly control DNA methylation raises the possibility that connections between protein acetylation and DNA methylation result in self-reinforcing epigenetic states.**

DNA is modified by methylation of cytosines in many higher organisms, including mammals, plants, and some fungi. DNA methylation can silence genes (see refs. 1 and 2) and may serve in genome defense systems (3, 4) and in the regulation of certain endogenous genes, such as genes subjected to genomic imprinting or dosage compensation in mammals (5). Although DNA methylation does not appear to interfere directly with transcription, it can indirectly prevent transcription initiation (6) or elongation (7, 8). How methylation exerts its repressive effect remains largely undefined, but proteins that bind specifically to methylated DNA have been identified (9), and methylation appears to cause assembly of an inactive form of chromatin (6, 10). Histones H3 and H4 are hypoacetylated on the heavily methylated inactive X chromosome (11–14) and hyperacetylated in the unmethylated "CpG islands" in animal genomes (15). In a study on sequences introduced into animal cells as episomes, it was found that 5-azacytidine (5-AC) and sodium butyrate, which cause hypomethylation of DNA and hyperacetylation of histones, respectively, could both relieve repression (16). Butyrate has pleiotropic effects at the high concentrations at which it must be used (see ref. 17), but this observation raised the possibility that methylation operates through an effect on histone acetylation, or *vice versa*. Recently, a potent direct inhibitor of histone deacetylases, (*R*)-trichostatin A (TSA) (18), was found to substitute for 5-AC to derepress silent, methylated rDNA genes in interspecific plant hybrids (19). Changes in methylation and/or acetylation in the rDNA, or at an undefined regulatory locus, may have caused the derepression. Effects on DNA methylation were not assessed. Two key questions are (*i*) Can DNA methylation affect histone acetylation? and (*ii*) Can acetylation affect DNA methylation? Either possibility could account for the observed correlations. If both occur, this should produce a self-reinforcing cycle that could account for stable epigenetic states.

The fungus *Neurospora crassa* offers an attractive system to investigate these possibilities. Most of the *Neurospora* genome is unmethylated and DNA methylation is nonessential (20, 21), but it is clear that DNA methylation can control some genes in this organism (8, 22, 23). Most specifically, we know that methylation of alleles of the *am* (glutamate dehydrogenase) and *mtr* (methyltryptophan-resistant) genes prevent transcription elongation (8). Inhibition of DNA methylation by the drug 5-AC or by the *dim-2* mutation, which prevents all methylation in *Neurospora*, activates methylated *am* and *mtr* alleles. Similarly, inhibition of methylation by using 5-AC or other means reactivates a methylated copy of the bacterial *hph* (hygromycin B phosphotransferase) gene (23) and causes repression of an allele of *am* whose expression depends on methylation of a transposon inserted in its upstream region (22). As a step to investigate the mechanism of methylation-dependent effects on gene expression, I investigated the effect of the histone deacetylase inhibitor TSA on expression of these genes. TSA was found to reverse the effects attributable to methylation. Analyses of DNA methylation demonstrated that TSA can cause selective loss of demethylation in *Neurospora*, implying that acetylation of histones or other proteins can somehow control DNA methylation.

MATERIALS AND METHODS

Strains. The following strains from our laboratory collection were used in this study: N220 (*am::Tad3-2 ure-2 mat a*) (22), N644 (am^{132} [(*am/hph/am*)$^{ec42\ pJI2}$]^{RIP77} *inl mat A*) (23), N669 (am^{RIP4} am^{RIPec4} *lys-1 mat a*), N672 (am^{RIP5} am^{RIPec5} *lys-1 mat A*), N617 (am^{RIP8MM} *mat a*), N676 (am^{RIP7} am^{ec7} *lys-1 mat A*) (24). The *am/hph/am* region of N644 was from pJI2, and consists of a direct repeat of the *am* gene separated by a 1.7-kb segment including the bacterial *hph* gene from pDH25 driven by the *Aspergillus nidulans trpC* promoter (25, 26). The am^{132} allele contains a deletion that removes all sequences homologous to the *am* probe and to *am* sequences introduced by transformation.

Cultures. Liquid cultures of *Neurospora*, inoculated with 5–10 × 10^4 fresh conidia per ml, were grown at 32°C with shaking in sucrose (1.5–2.0%) Vogel's medium (27) supple-

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Abbreviations: 5-AC, 5-azacytidine; TSA, trichostatin A; HAT, histone acetyltransferase; HDAC, histone deacetylase; RIP, repeat-induced point mutation; hyg, hygromycin.

mented with alanine and inositol to support growth of *am, inl* strains. TSA (Wako) was added to the medium immediately prior to inoculation. Plate tests were performed with solidified Vogel's medium containing sorbose (2%), fructose (0.05%), and glucose (0.05%) in place of sucrose to cause colonial growth. Glycine (20 mM) was included in some plates to tighten the selection for am^+ strains. Conidia were routinely plated in 5 ml of 0.7% agar on plates with 25 ml of 1.5% agar medium. TSA (1 μl of 10 mg/ml in dimethyl sulfoxide) was administered from 4-mm-diameter Whatman no. 1 paper discs placed in the middle of plates shortly after plating. For some experiments, an additional 5 ml of top agar with or without hygromycin B (hyg; 600 μg/ml; Calbiochem) was added after 18 hr at 32°C. Plates were typically photographed 40 hr later.

Southern Hybridizations. DNA was isolated from 1- to 3-day liquid cultures as previously described. DNA samples (1 μg) were digested for at least 4 hr with 10 units of restriction enzyme (NEB), fractionated on 1% agarose gels, transferred to nylon membranes, and probed sequentially as previously described (23). Probes were prepared by priming with random hexamers using the 2.6-kb *Bam*HI *am* fragment, the 650-bp *Xba*I–*Bam*HI upstream *am* fragment, the 1.0-kb *Cla*I Ψ_{63} fragment, the 9.2-kb *Kpn*I rDNA fragment, the 1.0-kb *Cla*I-*Bam*HI *hph* fragment, or a 2.5-kb *his-3* fragment (to verify that digests were complete; not shown).

RESULTS

The possibility that TSA would activate a gene repressed by DNA methylation was first tested with the bacterial gene *hph*, which is present as a single chromosomal copy in *N. crassa* strain N644 (23). This strain was derived from a transformant in which *hph* was unmethylated and conferred resistance to hyg (26). The approximately 1-kb gene, driven by the *A. nidulans trpC* promoter (see *Materials and Methods*), lies between two copies of the *Neurospora am* (glutamate dehydrogenase) gene. This allowed us to render *hph*, and the *trpC* promoter, methylated by induction of RIP in the flanking repeated *am* sequences (23). RIP is a sexual-phase-specific genome defense system that results in multiple G·C to A·T transition mutations in duplicated sequences (4, 28–31). Remaining cytosines are frequently methylated after the action of RIP and the methylation can extend into adjacent unmutated sequences, such as those of the *hph* gene flanked by mutated copies of *am*. We had identified progeny of the original transformant in which RIP had indeed caused heavy methylation of the two copies of *am* plus the intervening sequences, including *hph*. The *hph* gene was silenced in most strains (e.g., N644) but expression could be restored if methylation was inhibited by treatment with 5-AC or by limiting production of the methyl-group donor, *S*-adenosylmethionine (AdoMet), using a conditional mutation in the AdoMet synthetase gene (23). The effect of TSA on expression of the methylated *hph* gene was tested by plating conidia (asexual spores) of strain N644 in the presence or absence of TSA (10 μg = 33 nmol) applied to paper discs in the middle of the plates. Because hyg quickly kills strains not expressing *hph* (23), this drug was added 18 hr after plating the conidia. One day later, hyg-resistant colonies appeared near the source of TSA; no colonies were seen on the control plate without TSA (Fig. 1*A*). The density of colonies near the disc appeared equivalent to the density on a similar plate lacking hyg and TSA, suggesting that TSA caused derepression in virtually every colony. Other plate tests revealed that 20 nmol (5 μg) of 5-AC induced *hph* roughly as well as 33 nmol of TSA (Fig. 1*B*), although 5-AC inhibited growth considerably more than did TSA (data not shown). Interestingly, TSA and 5-AC together seemed to result in greater induction than either drug alone (Fig. 1*B*). To test the stability of the apparent derepression by TSA, colonies from plates with TSA and hyg, and colonies from control plates lacking the drugs, were picked and

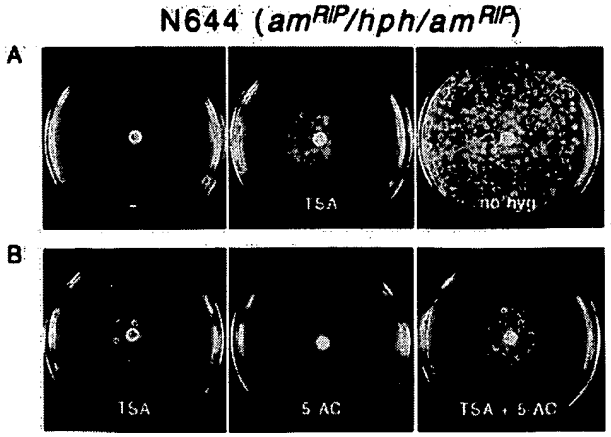

FIG. 1. Reactivation of silenced *hph* gene by TSA and 5-AC. *N. crassa* strain N644 (am^{132}, *inl*, $am^{RIP}/hph/am^{RIP}$, *mat A*) harbors a single copy of the *Escherichia coli hph* gene that was inactivated by methylation because of the action of RIP on flanking direct repeats of the *am* gene (23, 26). Sets *A* and *B* were from separate experiments using independent solutions and cultures. In each experiment shown, ≈1,000 conidia were plated on each of the plates, 1 μl of TSA (33 mM in dimethyl sulfoxide), 5-AC (20 mM), both, or neither (plates marked − and no hyg) was applied to the paper discs, and, except for the right-most plate in *A*, hyg was added in 0.7% agar medium after 17–18 hr at 32°C. Control plates with TSA or 5-AC but lacking hyg revealed slight inhibition of growth by TSA and somewhat greater inhibition by 5-AC (not shown).

tested on plates containing hyg but no TSA. Colonies from the TSA plus hyg plate, but not the control plate, grew vigorously on hyg medium, but showed loss of resistance after conidiation (data not shown). Thus TSA caused a long-term, but not permanent, derepression of the *hph* gene.

A case in which methylation affects gene expression in the opposite direction from that normally observed provided an attractive opportunity for a second genetic test of the effect of TSA on methylated DNA. Expression of the *am* gene in strain N220 relies somehow on methylation spanning the 5′ end of a LINE-like transposon, *Tad*, inserted upstream of the *am* basal promoter (22) (Fig. 2*B*). *am* expression is required for growth on minimal medium supplemented with glycine. Normally, ≈2% of N220 conidia plated on restrictive (glycine) medium form colonies (ref. 22 and data not shown). When methylation is prevented, however, by using either 5-AC or a strain harboring the *dim-2* mutation, which prevents all methylation in vegetative tissue of *Neurospora* (20), the *am* gene is fully silenced. Loss of methylation somehow allows *Tad* to silence *am*. Although the cause of this methylation is not known, it is apparently not a result of RIP (22).

The effect of TSA on *am* expression in strain N220 was tested by plating ≈400,000 conidia on permissive (alanine) or restrictive (glycine) media and then adding TSA (10 μg) to the center of the dishes, as before. A slight inhibition of growth was detected near the source of the TSA on alanine medium, but the strain produced essentially confluent growth, as expected (Fig. 2*A*). TSA did not appreciably inhibit a wild-type strain on either alanine or glycine medium (not shown). In striking contrast, growth of N220 on glycine medium was limited to the region of the plate at least 2.5 cm from the source of the TSA. Thus the deacetylase inhibitor accentuated the inhibitory effect of *Tad* on *am* expression, as occurs when DNA methylation is prevented (22).

The effects of TSA on expression of *hph* and *am* suggested that histone hypoacetylation and DNA methylation may operate in a common silencing pathway. One possibility was that the silencing resulted from hypoacetylation directed by the DNA methylation. It was also possible that hypoacetylation

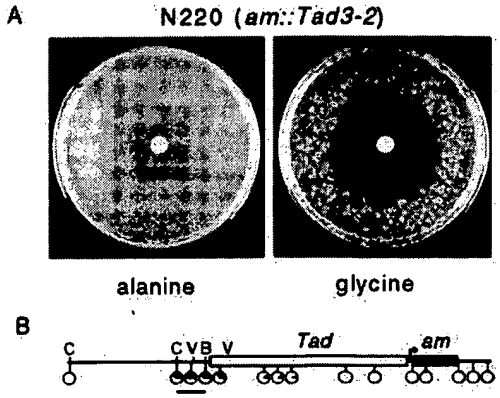

FIG. 2. TSA accentuates silencing of the *am* gene caused by the adjacent transposon *Tad*. (*A*) Approximately 4 × 10^5 conidia of *N. crassa* strain N220 (*am::Tad3–2, ure-2, mat a*) were plated on permissive (alanine) or restrictive (glycine) sorbose plates. One microliter of TSA (33 mM in dimethyl sulfoxide) was applied to the paper discs and the plates were incubated 3 days at 32°C. The difference in colony density in the portions of the plates not affected by TSA reflects the ratio of Am^+ and Am^- colonies characteristic of this strain. (*B*) Map of *am::Tad3–2* region of strain N220. *Tad* (open rectangle) is inserted 70 bp upstream of the transcription start sites (arrow) of *am* (black rectangle). The approximate methylation status of 15 sites in the region, as determined in a previous study (22), is depicted in black in pie charts placed close to the sites examined. Those sites for *Bam*HI (B), *Cla*I (C), and *Eco*RV (V) that are relevant to Fig. 5 are indicated. The bar beneath the map represents a 650-bp *Xba*I–*Bam*HI fragment used as a probe for the blot shown in Fig. 5.

triggered DNA methylation, which then more directly affected gene expression. I therefore investigated whether TSA affected DNA methylation. The methylation state of the am^{RIP}/*hph*/am^{RIP} region was examined in N644 grown in nonselective medium containing up to 1 μg/ml (3.3 μM) TSA, a concentration that retarded growth somewhat under the conditions of the experiment (Fig. 3*A*). Methylation was assessed by comparing *Sau*3AI and *Dpn*II digests by Southern hybridization. *Sau*3AI fails to cut DNA when the C in its recognition site (GATC) is methylated, whereas its isoschizomer *Dpn*II is not inhibited by cytosine methylation. A substantial reduction in methylation in the am^{RIP}/*hph*/am^{RIP} region was caused by growth in 3.3 μM TSA, as shown by the fact that *Sau*3AI gave nearly complete digestion of *am* and *hph* sequences (Fig. 3*B* and data not shown).

Although this finding suggested that TSA may have inhibited methylation, it seemed possible that the reduced methylation was simply the result of inhibited growth, because young cultures of *Neurospora* show some reduction in overall methylation (32). Therefore DNA from N644 cultures grown for longer periods of time with and without TSA were examined. Derepression of *hph* was also tested in the same set of cultures by challenging them to grow in the liquid medium after addition of hyg. Cultures with 0.1 μg/ml (0.33 μM) TSA, or less, failed to grow appreciably in the presence of hyg (data not shown). In contrast, cultures supplemented with 3.3 μM TSA grew well in hyg medium. Although all cultures with this level of TSA showed somewhat retarded growth initially, as in the previous experiment, by 46 hr they appeared to have "caught up" to the cultures without TSA. This observation was confirmed by measuring tissue weights (Fig. 4 legend and data not shown). Most interestingly, DNA prepared from mature (2- and 3-day) cultures grown nonselectively in the presence of TSA showed marked reduction in methylation in the am^{RIP}/*hph*/am^{RIP} region (Fig. 4*A*), as observed with the younger culture (Fig. 3). No difference in methylation was evident between the 2- and 3-day cultures, reinforcing the conclusion that the reduction in methylation observed here and in the

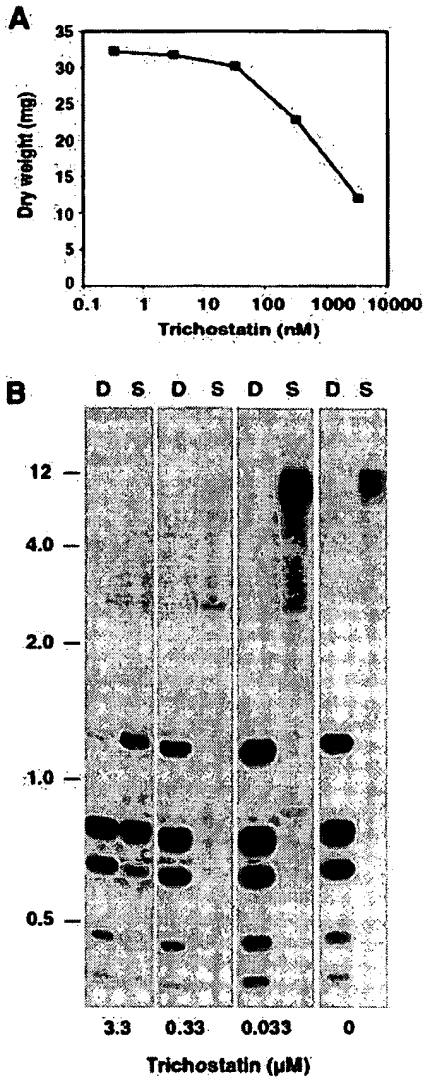

FIG. 3. Effect of TSA on growth and DNA methylation. (*A*) Liquid cultures inoculated with *N. crassa* strain N644 (7 × 10^4 conidia per ml) were supplemented with up to 1 μg/ml (3.3 μM) TSA and grown 27 hr. The untreated culture yielded 32.8 mg of dry tissue. (*B*) Southern hybridization of selected samples from the cultures. Samples of DNA were prepared and digested with *Dpn*II (D) or *Sau*3AI (S) and probed for *am* sequences. Stronger signals in the 0.33 μM lanes is partially due to 2× heavier loadings of DNA in these lanes. The positions of selected size standards (kb) are indicated.

previous experiment was not somehow due to differences in growth. DNA from a culture grown in TSA and hyg showed the greatest reduction in methylation (Fig. 4*A*), presumably because selection for hyg favored cells with reduced methylation of *hph* (23).

The marked reduction in methylation caused by TSA in the am^{RIP}/*hph*/am^{RIP} region was particularly surprising because no change in overall methylation was apparent from inspection of the ethidium bromide-stained genomic DNA samples (Fig. 4*B*). We had previously noted that comparisons of *Sau*3AI and *Dpn*II (or *Mbo*I) digests of *N. crassa* DNA provide a simple indicator of overall methylation (33). Thus it seemed possible that TSA caused a highly selective loss of methylation in the genome, in contrast to the rather uniform reduction in methylation achieved by 5-AC treatment, by use of any of several mutants defective in methylation (refs. 20, 32, and 34; H. Foss, C. Roberts, and E.S., unpublished results; and A. Hagemann, M. Freitag, and E.S., unpublished results) or by limiting

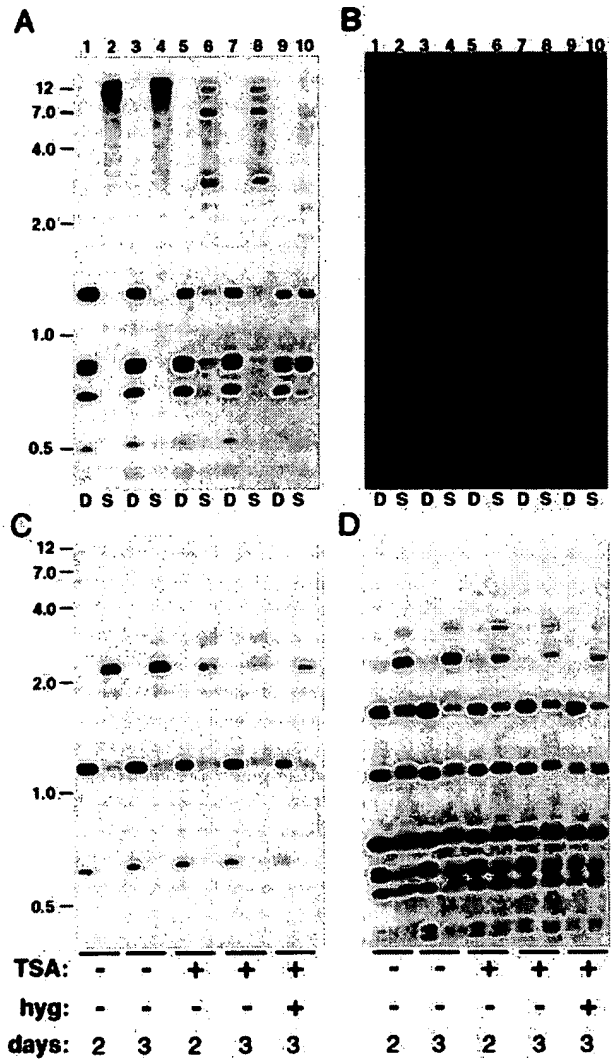

FIG. 4. TSA causes selective hypomethylation of DNA. Strain N644 was grown for 2 or 3 days, as indicated, with or without TSA (1 μg/ml; lanes 5–10) and hyg (added to a concentration of 0.1 mg/ml 17 hr after inoculation; lanes 9 and 10). The dry weights of the cultures illustrated were, from left to right, 31.1 mg, 38.5 mg, 37.5 mg, 37.3 mg, and 32.8 mg. DNA was isolated and analyzed by digestion with *Dpn*II (D) or *Sau*3AI (S) and by probing for *am* (*A*), Ψ_{63} (*C*), rDNA (*D*), or *hph* (not shown). (*B*) Total genomic DNA visualized by staining with ethidium bromide. The positions of selected size standards (kb) are indicated.

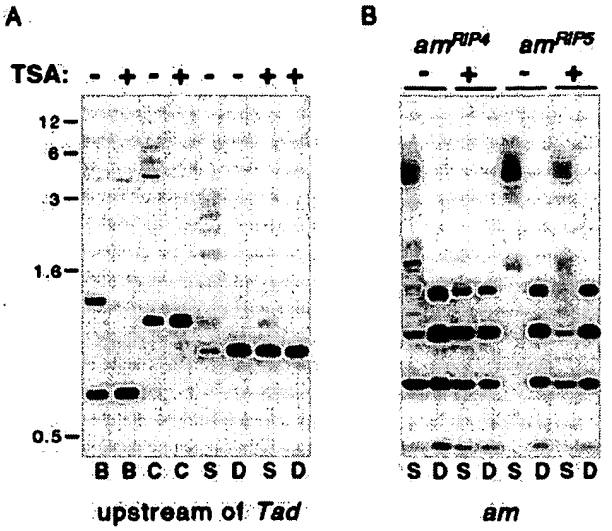

FIG. 5. TSA-induced hypomethylation of *am*::*Tad3–2* and am^{RIP} alleles. Strains containing *am*::*Tad3-2* (N220), am^{RIP4} and am^{RIPec4} [N669 (24)] or am^{RIP5} and am^{RIPec5} [N672 (24)] were grown from conidia for 2 days in the presence or absence of 1 μg/ml TSA, as indicated. (*A*) DNA samples of N220 were digested with *Eco*RV (which is not inhibited by cytosine methylation) plus *Bam*HI (B), *Bsp*106I (an isoschizomer of *Cla*I; C), *Sau*3AI (S), or *Dpn*II (D) and probed for sequences upstream of *Tad* (see Fig. 2*B*). The positions of selected size standards (kb) are indicated. (*B*) DNA of N669 or N672 was digested with *Sau*3AI or *Dpn*II and probed sequentially for *am*, Ψ_{63} (not shown), and rDNA (not shown).

methylation by using strains harboring mutations in genes required for *S*-adenosylmethionine biosynthesis (20, 34). To test directly whether TSA caused differential hypomethylation, the blot shown was reprobed for two other regions known to be methylated in the *N. crassa* genome. These were Ψ_{63}, a 5S rRNA pseudogene interrupted by a transposon that has been inactivated by RIP (20, 35), and the tandemly repeated rRNA genes (36). Hybridization results revealed that neither Ψ_{63} (Fig. 4*C*) nor rDNA (Fig. 4*D*) sequences were hypomethylated. Thus the histone deacetylase inhibitor caused striking hypomethylation in the am^{RIP}/*hph*/am^{RIP} region but caused no apparent change in methylation of the other regions.

The effect of TSA on methylation was also examined in the upstream *am*/*Tad* boundary in strain N220 and in several additional alleles of *am* that were methylated as a result of RIP. Digestion of N220 DNA with *Bam*HI, Bsp106I, or *Sau*3AI revealed TSA-induced hypomethylation at the upstream *am*/*Tad* boundary (Fig. 5*A*), as found in the am^{RIP}/*hph*/am^{RIP} region of N644. The am^{RIP}/*hph*/am^{RIP} construct of N644 was built using only sequences downstream of the *Bam*HI site (Fig. 2*B*); thus the methylated upstream *am*/*Tad* sequences include less than 180 bp found in strain N644.

Strains bearing *am* alleles inactivated by RIP showed similar results. Several strains examined each contain two am^{RIP} alleles, one at the native *am* locus and another at the unlinked site where the 2.6-kb *Bam*HI *am* fragment that triggered RIP had integrated (24, 29, 37). Strain N669 was chosen because the methylation of both *am* sequences (am^{RIP4} and am^{RIPec4}) in this strain appears to be dependent on maintenance methylation. That is, if the methylation of these alleles is removed by treatment with 5-AC, or if one of these alleles is isolated and reintroduced into *Neurospora* in an unmethylated state, the methylation is not reestablished (24). In contrast, in strain N672, the am^{RIP5} and am^{RIPec5} alleles become methylated *de novo*, as is most common for sequences bearing moderate or heavy damage by RIP (4). Interestingly, both of the am^{RIP} alleles in both strains N669 and N672 showed striking reduction of methylation in response to TSA treatment (Fig. 5*B*). The alleles that do not trigger *de novo* methylation and therefore must depend on maintenance methylation (am^{RIP4} and am^{RIPec4}) were most affected. Curiously, in strain N672, the allele at the native *am* locus appeared more sensitive to TSA than the ectopic allele, as indicated by the relatively weak signal of the *Sau*3AI fragment diagnostic of the ectopic copy [1.4-kb fragment matching the largest *Dpn*II fragment (24)]. A strain (N617) containing only a single *am* allele (am^{RIP8}), at its native locus, also showed dramatic reduction in methylation (data not shown). Reprobings of the blot with Ψ_{63} and rDNA (data not shown) revealed no apparent loss of methylation from these regions, as with strain N644 (Fig. 4).

DISCUSSION

The exposed N-terminal tails of each of the four core histones are subject to a variety of posttranslational modifications that may affect chromatin function. Early evidence that acetylation

of lysines in the tails is associated with gene expression has been consistently supported and extended (see refs. 38–40). The general picture that has emerged is that hyperacetylation is a prerequisite for transcription, whereas hypoacetylation can result in transcriptional repression (see refs. 41–43). Several transcription factors and transcriptional coactivators have been demonstrated to possess histone acetyltransferase (HAT) activity (44, 45), and a number of transcriptional repressors have been shown to recruit histone deacetylases (HDACs) (46–51). Studies with antibodies specific to histones acetylated at particular sites (52) or with probes for sequences in hyper- or hypoacetylated chromatin (53) revealed gross variation in histone acetylation in different chromosomal regions, presumably reflecting differential availability of HATs and HDACs. DNA sequences associated with epigenetic silencing, such as on the inactive X chromosome of mammals (11–14), in heterochromatin of insects (54), in the silent mating type genes in yeasts (55, 56), and in the centromere regions of fission yeast (57), are typically associated with hypoacetylated histones. Methylated sequences in animals have also been found associated with hypoacetylated histones (15).

Direct evidence that histone acetylation can affect gene expression came from studies in which genes for HDACs were mutated (58) or inhibited with drugs (16, 17, 19, 49, 57, 59, 60). Curiously, two cases were found in which genes could be activated by using either an inhibitor of DNA methylation (5-AC) or an inhibitor of HDACs (butyrate or TSA). In the first, Hsieh (16) showed that butyrate could enhance expression of a methylated episome transfected into human cells. No effect on methylation was detected. In the second study, both butyrate and a specific inhibitor of HDACs, TSA, were found to substitute for 5-AC to derepress silent, methylated rDNA genes in interspecific plant hybrids (19). Effects on DNA methylation were not assessed. Thus, changes in methylation and/or acetylation in the rDNA, or at an undefined regulatory locus, may have caused the derepression. Nevertheless, these findings raised two possibilities: the repressive effect typical of DNA methylation may be mediated by deacetylation of histones, or deacetylation may lead to DNA methylation. These possibilities were investigated by using genes in *Neurospora* whose expression is known to be negatively or positively controlled by DNA methylation: a bacterial transgene (*hph*) repressed by methylation (23), an allele of the *Neurospora am* gene that is controlled indirectly by methylation of a transposon (*Tad*) inserted upstream of the *am* basal promoter (22), and several methylated alleles of *am* generated by RIP (24). TSA treatment induced expression of *hph* and silenced expression of the *am* allele downstream of *Tad*. Surprisingly, DNA methylation was dramatically reduced in the *hph* and *Tad/am* regions, as well as in all am^{RIP} alleles examined. Thus, the changes in gene expression may have resulted directly from histone hyperacetylation, as has been observed in other systems, or may have resulted indirectly from an effect of hyperacetylation on methylation. Although it is conceivable that TSA affected a process other than histone acetylation, TSA is a direct, noncompetitive inhibitor of HDACs and no other effects of the drug have yet been found (18). As far as I know, this is the first indication that DNA methylation may depend, directly or indirectly, on the acetylation state of histones. Interestingly, TSA did not affect methylation of other genomic sequences examined, including the nucleolar rDNA and a transposon inactivated by RIP.

What is the most straightforward interpretation of these results? In every system examined, TSA has been found to cause hyperacetylation of the core histones, apparently because their state of acetylation reflects the balance of HATs and HDACs working in opposition (18, 19, 57, 59, 61). Variation in the distribution of HATs and HDACs in different chromosomal regions should result in regional differences in the degree of hyperacetylation in response to TSA, but this possibility has not been carefully examined. In principle, the observed loss of methylation caused by TSA in our system could have resulted either from hyperacetylation of the methylated regions or from hyperacetylation elsewhere in the genome. For example, if a hypothetical negative regulator of a DNA methyltransferase were activated by increased acetylation in the vicinity of its gene, this might lead to decreased DNA methylation. This is not the most economical model, however, especially considering that TSA strongly affected methylation in some chromosomal regions but left other areas unaffected. There are several possible explanations for the differential effect of TSA on DNA methylation. TSA may not have inhibited the action of HDACs in all regions. Alternatively, deacetylation may have been inhibited globally but only some regions had access to HATs and were therefore reacetylated. Consistent with this line of reasoning, it has recently been shown that TSA activates the WAF1/Cip1 promoter in a human cell line through Sp1 sites (59). This is particularly interesting in light of evidence that deletion or mutations in Sp1 sites of the mouse and hamster *aprt* genes lead to methylation of their CpG islands (62–64). Thus, it does not seem unreasonable to suppose that in our system the *am* enhancer region upstream of *Tad3-2* would recruit a HAT and thereby increase acetylation in the region after TSA treatment, whereas the Ψ_{63} pseudogene, with its heavily mutated transposon (35), would lack sites to which HATs are recruited and thus remain hypoacetylated. It is worth noting in this context that the upstream region of the *Neurospora am* gene includes a CCAAT site that binds a protein (AAB) equivalent to HAP5 of yeast, which is a member of an activation complex dependent on the HAT encoded by GCN5 (65, 66). The distribution of regulatory sites and DNA methylation in rDNA is not yet well defined in *Neurospora*, but it is interesting that butyrate was not found to influence the gross acetylation level of histones in rDNA of human chromosomes (53).

The observed loss of methylation could be a direct or indirect result of TSA-induced hyperacetylation. It is possible that hyperacetylation released transcription in some chromosomal regions and the transcription, rather than hyperacetylation, *per se*, inhibited DNA methylation. We know that absence of transcription is not sufficient to trigger methylation and that methylation of *am* prevents transcription elongation (8), but it is conceivable that activation by hyperacetylation could overcome this effect. A second possibility consistent with the observations is that acetylation directly controls DNA methylation. Hypoacetylation could trigger DNA methylation or hyperacetylation could inhibit methylation (or both). Either DNA methylation or hypoacetylation could be responsible for repression of transcription in this model. The observation of interchangeable effects of 5-AC and TSA implies, however, that hypoacetylation cannot be solely responsible for repression unless the state of methylation feeds back on the state of acetylation. Recent suggestions that methyl-DNA binding proteins may recruit HDACs are consistent with this possibility (67, 68). If methylated sequences recruit HDACs, which then cause deacetylation, and deacetylation promotes methylation, this should produce a rather stable repressed state. This self-reinforcing epigenetic state could account for observed maintenance of methylation, including maintenance of heterogeneous methylation at nonsymmetrical sites (69). To distinguish between these models it will be necessary to determine whether transcription is required for TSA-induced changes in methylation and whether DNA methylation can directly affect histone acetylation. A variety of studies will be required to discover all the connections between modifications of DNA and chromatin and between these modifications and gene expression.

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1. Bird, A. (1992) *Cell* **70,** 5–8.
2. Kass, S. U., Pruss, D. & Wolffe, A. P. (1997) *Trends Genet.* **13,** 444–449.
3. Yoder, J. A., Walsh, C. P. & Bestor, T. H. (1997) *Trends Genet.* **13,** 335–340.
4. Selker, E. U. (1997) *Trends Genet.* **13,** 296–301.
5. Jaenisch, R. (1997) *Trends Genet.* **13,** 323–329.
6. Buschhausen, G., Wittig, B., Graessmann, M. & Graessmann, A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1177–1181.
7. Barry, C., Faugeron, G. & Rossignol, J.-L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4557–4561.
8. Rountree, M. R. & Selker, E. U. (1997) *Genes Dev.* **11,** 2383–2395.
9. Tate, P. H. & Bird, A. P. (1993) *Curr. Biol.* **3,** 226–231.
10. Keshet, I., Lieman-Hurwitz, J. & Cedar, H. (1986) *Cell* **44,** 535–543.
11. Jeppesen, P. & Turner, B. M. (1993) *Cell* **74,** 281–289.
12. O'Neill, L. P. & Turner, B. M. (1995) *EMBO J.* **14,** 3946–3957.
13. Keohane, A. M., O'Neill, L. P., Belyaev, N. D., Lavender, J. S. & Turner, B. M. (1996) *Dev. Biol.* **180,** 618–630.
14. Boggs, B. A., Connors, B., Sobel, R. E., Chinault, A. C. & Allis, C. D. (1996) *Chromosoma* **105,** 303–309.
15. Tazi, J. & Bird, A. (1990) *Cell* **60,** 909–920.
16. Hsieh, C. L. (1994) *Mol. Cell. Biol.* **14,** 5487–5494.
17. Kruh, J. (1982) *Mol. Cell. Biochem.* **42,** 65–82.
18. Yoshida, M., Kijima, M., Akita, M. & Beppu, T. (1990) *J. Biol. Chem.* **265,** 17174–17179.
19. Chen, Z. J. & Pikaard, C. S. (1997) *Genes Dev.* **11,** 2124–2136.
20. Foss, H. M., Roberts, C. J., Claeys, K. M. & Selker, E. U. (1993) *Science* **262,** 1737–1741.
21. Foss, H. M., Roberts, C. J., Claeys, K. M. & Selker, E. U. (1995) *Science* **267,** 316.
22. Cambareri, E. B., Foss, H. M., Rountree, M. R., Selker, E. U. & Kinsey, J. A. (1996) *Genetics* **143,** 137–146.
23. Irelan, J. T. & Selker, E. U. (1997) *Genetics* **146,** 509–523.
24. Singer, M. J., Marcotte, B. A. & Selker, E. U. (1995) *Mol. Cell. Biol.* **15,** 5586–5597.
25. Cullen, D., Leong, S. A., Wilson, L. J. & Henner, D. J. (1987) *Gene* **57,** 21–26.
26. Irelan, J. T., Hagemann, A. T. & Selker, E. U. (1994) *Genetics* **138,** 1093–1103.
27. Davis, R. H. & De Serres, F. J. (1970) *Methods Enzymol.* **17A,** 47–143.
28. Selker, E. U., Cambareri, E. B., Jensen, B. C. & Haack, K. R. (1987) *Cell* **51,** 741–752.
29. Selker, E. U. & Garrett, P. W. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6870–6874.
30. Cambareri, E. B., Jensen, B. C., Schabtach, E. & Selker, E. U. (1989) *Science* **244,** 1571–1575.
31. Selker, E. U. (1990) *Annu. Rev. Genet.* **24,** 579–613.
32. Foss, H. M., Roberts, C. J. & Selker, E. U. (1998) *Mol. Gen. Genet.*, in press.
33. Selker, E. U., Richardson, G. A., Garrett-Engele, P. W., Singer, M. J. & Miao, V. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 323–329.
34. Roberts, C. J. & Selker, E. U. (1995) *Nucleic Acids Res.* **23,** 4818–4826.
35. Margolin, B. S., Garrett-Engele, P. W., Stevens, J. N., Yen-Fritz, D., Garrett-Engele, C., Metzenberg, R. L. & Selker, E. U. (1998) *Genetics*, in press.
36. Perkins, D. D., Metzenberg, R. L., Raju, N. B., Selker, E. U. & Barry, E. G. (1986) *Genetics* **114,** 791–817.
37. Selker, E. U., Fritz, D. Y. & Singer, M. J. (1993) *Science* **262,** 1724–1728.
38. Csordas, A. (1990) *Biochem. J.* **265,** 23–38.
39. Wade, P. A., Pruss, D. & Wolffe, A. P. (1997) *Trends Biochem. Sci.* **22,** 128–132.
40. Grunstein, M. (1997) *Nature (London)* **389,** 349–352.
41. Hampsey, M. (1997) *Trends Genet.* **13,** 427–429.
42. Pazin, M. J. & Kadonaga, J. T. (1997) *Cell* **89,** 325–328.
43. Hebbes, T. R., Thorne, A. W., Clayton, A. L. & Crane-Robinson, C. (1992) *Nucleic Acids Res.* **20,** 1017–1022.
44. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. & Allis, C. D. (1996) *Cell* **84,** 843–851.
45. Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., Nakatani, Y. & Allis, C. D. (1996) *Cell* **87,** 1261–1270.
46. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., *et al.* (1997) *Nature (London)* **387,** 43–48.
47. Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber Agus, N. & DePinho, R. A. (1997) *Nature (London)* **387,** 49–55.
48. Hassig, C. A., Fleischer, T. C., Billin, A. N., Schreiber, S. L. & Ayer, D. E. (1997) *Cell* **89,** 341–347.
49. Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E. & Eisenman, R. N. (1997) *Cell* **89,** 349–356.
50. Kadosh, D. & Struhl, K. (1997) *Cell* **89,** 365–371.
51. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y. & Evans, R. M. (1997) *Cell* **90,** 569–580.
52. Turner, B. M., Birley, A. J. & Lavender, J. (1992) *Cell* **69,** 375–384.
53. Breneman, J. W., Yau, P. M., Swiger, R. R., Teplitz, R., Smith, H. A., Tucker, J. D. & Bradbury, E. M. (1996) *Chromosoma* **105,** 41–49.
54. Bone, J. R., Lavender, J., Richman, R., Palmer, M. J., Turner, B. M. & Kuroda, M. I. (1994) *Genes Dev.* **8,** 96–104.
55. Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D. & Broach, J. R. (1993) *Genes Dev.* **7,** 592–604.
56. Braunstein, M., Sobel, R. E., Allis, C. D., Turner, B. M. & Broach, J. R. (1996) *Mol. Cell. Biol.* **16,** 4349–4356.
57. Ekwall, K., Olsson, T., Turner, B. M., Cranston, G. & Allshire, R. C. (1997) *Cell* **91,** 1021–1032.
58. Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M. & Grunstein, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14503–14508.
59. Sowa, Y., Orita, T., Minamikawa, S., Nakano, K., Mizuno, T., Nomura, H. & Sakai, T. (1997) *Biochem. Biophys. Res. Commun.* **241,** 142–150.
60. Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y. & Townes, T. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5798–5803.
61. Sheridan, P. L., Mayall, T. P., Verdin, E. & Jones, K. A. (1997) *Genes Dev.* **11,** 3327–3340.
62. Brandeis, M., Frank, D., Keshet, I., Siegfried, Z., Mendelsohn, M., Nemes, A., Temper, V., Razin, A. & Cedar, H. (1994) *Nature (London)* **371,** 435–438.
63. Macleod, D., Charlton, J., Mullins, J. & Bird, A. P. (1994) *Genes Dev.* **8,** 2282–2292.
64. Mummaneni, P., Walker, K. A., Bishop, P. L. & Turker, M. S. (1995) *J. Biol. Chem.* **270,** 788–792.
65. Chen, H., Crabb, J. W. & Kinsey, J. A. (1998) *Genetics* **148,** 123–130.
66. Georgakopoulos, T. & Thireos, G. (1992) *EMBO J.* **11,** 4145–4152.
67. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N. & Bird, A. (1998) *Nature (London)* **393,** 386–389.
68. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J. & Wolffe, A. P. (1998) *Nat. Genet.* **19,** 187–191.
69. Hagemann, A. T. & Selker, E. U. (1996) in *Control and Function of DNA Methylation in Neurospora crassa,* eds. Russo, V. E. A., Martienssen, R. A. & Riggs, A. D. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 335–344.